FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

The Patent Rules, 2006

Complete Specification

(See section 10 and rule 13)

A COMPOUND FOR THE DETECTION OF BIO-THIOLS AND PROCESS FOR PREPARATION THEREOF

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH
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The following specification particularly describes the nature of this invention and the manner in which it is to be performed

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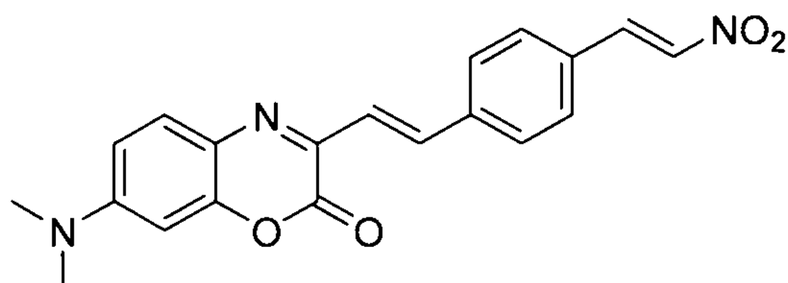
FIELD OF THE INVENTION:

The present invention relates to a novel compound of formula (I) for the detection of bio-thiols in aqueous medium. More particularly, the present invention relates to a compound of formula (I), process for preparation thereof and use of compound of formula (I) for detecting bio-thiols. The present invention further relates to a kit comprising compound of formula (I).



Formula I

BACKGROUND OF THE INVENTION:

Cysteine (Cys), Homocysteine (Hcy), and Glutathione (GSH) are the most important biological thiols, as they play essential roles for regulating the redox balance of various biological processes. Cys and Hcy are product of intracellular metabolism of methionine. The intracellular pool of Cys is relatively much smaller than the metabolically active pool of GSH in cells. It has been argued that Cys is generally the limiting amino acid for GSH synthesis in humans. Abnormal level of biothiols associated with various dysfunctions and diseases. Deficiency of Cys induces various diseases such as, slow growth, hair depigmentation, liver damage, skin lesions, hematopoiesis decrease, leukocyte loss, psoriasis, etc. Moreover Hcy is linked to different clinical conditions including Alzheimer's disease, schizophrenia, renal disease, cardiovascular disease, and diabetes. Hyper-homocysteinemia, abnormal level of Hcy in blood, is associated with premature cerebral, peripheral and coronary vascular disease. Increase in the supply of Cys or its precursors (e.g., cystine and N-acetylcysteine) via oral or intravenous intervention improves GSH synthesis and prevents GSH deficiency in humans under various nutritional and pathological conditions, such as protein malnutrition, adult respiratory distress syndrome, HIV and AIDS. Thus, effective quantification of biothiols in

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biological medium is expected to assist in early diagnosis of such crucial diseases. Accordingly, considerable efforts have been made for developing efficient reagent and appropriate methodology for quantitative detection of biothiols in physiological condition and in biofluids.

Numerous analytical methods have been employed for detection of biothiols which include high-performance liquid chromatography (HPLC), electrochemical assay, capillary electrophoresis and mass spectrometry. Most of these methods involve tiresome sample preparation and are not suitable for mapping distribution of either of these biothiols in live cells or tissues. Considering such limitations, reagents that allow fluorescence ON response either on binding or reaction with these biothiols have significance for developing efficient methodology(ies) that are relevant for clinical diagnosis, cell biology and assessing analytical samples. Fluorescence based methodology also provides high sensitivity and reliability. Larger Stokes shift, high emission quantum yield and emission at longer wavelength are the desired criteria for using a reagent for imaging application. Benzoxazine dyes are important class of dyes as they show very high stokes shift in comparison to the parent coumarins owing to their large dipole moment in excited state. Large stokes shift minimizes self-absorption and background noise due to auto-fluorescence while using the reagent for in-vitro or in-vivo imaging applications.

Reference may be made to an article entitled ''A Highly Selective Fluorescence Turn-on Sensor for Cysteine/Homocysteine and Its Application in Bioimaging'' by M. Zhang et al. published in *J. Am. Chem. Soc.,* 2007, 129, 10322-10323 have demonstrated a new fluorescence probe for imaging Cys/Hcy in living and fixed cells.

Reference may be made to an article entitled ''A Two-Photon Fluorescent Probe for Thiols in Live Cells and Tissues'' by J. H. Lee et al. published in *J. Am. Chem. Soc.,* 2010, 132, 1216–1217 *developed* a two-photon fluorescent probe that can detect thiols in live cells and living

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tissues at a 90−180 μm depth without interference from other biologically relevant species by two-photon microscopy.

Reference may be made to an article entitled ''A squaraine-based red emission off–on chemosensor for biothiols and its application in living cells imaging'' by X.D. Liu et al. published in *Org. Biomol. Chem.*, 2013, 11, 4258–4264 reports a squaraine-based red emission off–on for the detection biothiols and its application in living cells imaging.

Reference may be made to an article entitled "A Cysteine-Specific Fluorescent Switch for Monitoring Oxidative Stress and Quantification of Aminoacylase-1 in Blood Serum" by H.A. Anila et al. published in *Anal. Chem.*, 2016, 88, 12161−12168 reports a new coumarin derivative for specific and efficient chemodosimetric detection of cysteine.

Reference may be made to an article entitled "A fluorescent probe for specific detection of cysteine in the lipid dense region of cells" by F. Ali et al. published in *Chem. Commun.*, 2015,51, 16932-16935 reports a cysteine specific chemodosimetric reagent for imaging of endogenous cysteine, localized in the lipid dense region of the live Hct116 cells.

Reference may be made to an article entitled " A novel fluorescence probe for estimation of cysteine/histidine in human blood plasma and recognition of endogenous cysteine in live Hct116 cells" by U. Reddy et al. published in *Chem. Commun.*, 2014, 50, 9899-9902 reports a Cu(II)-complex based "Turn-On" luminescence probe for specific detection of endogenous Cys in live Hct116 cells and Cys present in human blood plasma without any interference from other amino acids, especially GSH and Hcy.

Reference may be made to an article entitled ''New Chemodosimetric Reagents as Ratiometric Probes for Cysteine and Homocysteine and Possible Detection in Living Cells and in Blood Plasma'' by P. Das et al. published in *Chem. Eur. J.* 2012, 18, 15382 – 15393 developed new ratiometric dual probes (electronic/fluorescent) for the detection of Cys/Hcy.

Reference may be made to an article entitled ''A cysteine sensor based on a gold nanoparticle–iron phthalocyanine modified graphite paste electrode'' by M.N. Abbas et al. published in *Anal. Methods,* 2015,7, 2529-2536 reports a gold nanoparticle (AuNP)–iron(III) phthalocyanine (FePc) modified graphite electrochemical sensor for the sensitive and selective detection of cysteine.

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Reference may be made to EP 2674759 B1, Jan 6, 2016, which reports Molecularly imprinted conducting polymer film based aqueous amino acid sensors.

Reference may be made to an article entitled ''A highly selective fluorescence sensor for cysteine/homocysteine and its application in bioimaging'' by T. Liu et al. published in *RSC Adv.,* 2015,5, 28713-28716 reports a squaraine based near-infrared probe for the detection of Cys/Hcy over other amino acids and GSH.

Reference may be made to an article entitled "A reagent for specific recognition of cysteine in aqueous buffer and in natural milk: imaging studies, enzymatic reaction and analysis of whey protein" by Anila H. A., Upendar Reddy G., Firoj Ali,a Nandaraj Taye, Samit Chattopadhyay and Amitava Das, Chem. Commun., 2015, 51, 15592 which reported a new chemodosimetric probe (L) for specific recognition of cysteine (Cys) in aqueous buffer and in whey protein isolated from fresh cow's milk. A luminescence based methodology for estimation of Cys released from a commercially available Cys-supplement drug by aminoacylase-1 in live cells is reported.

Considering this opportunity, for the first time we are demonstrating a fluorescent OFF-ON based molecular formula for the specific detection and quantification of both Cys and Hcy with excellent sensitivity. Under physiological condition the nitroolefin based benzooxazine derivative (Formula I) react only with Cys and Hcy by Michael addition reaction pathway to yield a compound with altered intramolecular charge transfer responses and a significant enhancement in emission quantum yield due to interruption of PET process. Interestingly, this probe showed luminescence ON response on reaction with proteins that are known to have Cys residue with free sulphydryl group and could further be used for detection of HSA in human urine.

OBJECTIVE OF THE INVENTION:

The main objective of the present invention is to provide a novel compound of formula (I).

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Another objective of the present invention is to provide a process for the preparation of compound of formula (I).

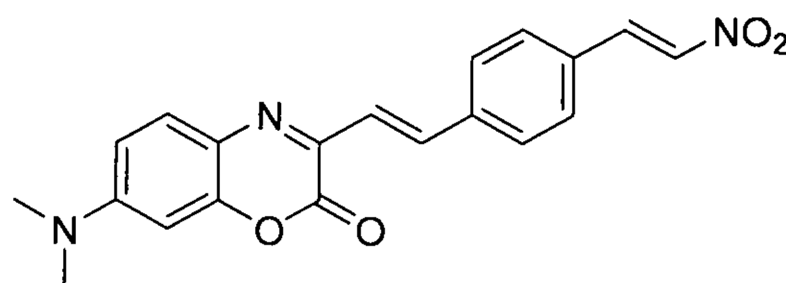
Another objective of the present invention is to use (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde as a starting material for the preparation of compound of formula (I).

Yet another objective of the present invention is to provide use of compound of formula (I) for the detection of bio-thiols (cysteine and homocysteine).

Still another objective of the present invention is to provide a kit for the detection of human serum albumin in urine comprising compound of formula (I).

SUMMARY OF THE INVENTION:

Accordingly, the present invention provides a compound of formula (I);



Formula I

In another embodiment, the compound of formula (I), used for the detection of cystine and homocystine.

In another embodiment, the compound of formula (I), is used to bind free SH group (thiol group) of SA (serum albumin) with increase in emission which detect SA in urine.

In another embodiment, the present invention provides a process for the preparation of compound of formula I comprising the steps of:

(i) dissolving (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl) benzaldehyde in nitromethane at a temperature in the range of 50-100°C;

(ii) adding ammonium acetate to the solution obtained in step (i) with stirring for 3-9 hours;

(iii) cooling the reaction mixture obtained in step (ii) to room temperature;

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(iv) washing the solid obtained in step (iii) to obtain the residue;

(v) extracting the residue obtained in step (iv) with water and organic solvent and

(vi) drying out the organic solvent layer to obtain the compound of formula (I).

In a preferred embodiment, the washing is performed using water and diethyl ether.

In a preferred embodiment, the organic solvent is selected from the group consisting of dicholoromethane, chloroform and ethyl acetate.

In another embodiment, the present invention provides a process for the preparation of compound of formula (I). To prepare Formula (I), 160 mg (0.5 mmol) of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde was dissolved in 10 ml of nitromethane. To it 385 mg (5 mmol) of ammonium acetate was added to it and heated at 80 °C for 4 h. On cooling to room temperature precipitate appeared, which was washed with water and then with 10 ml of diethylether. Residue was further extracted from DCM/water and the DCM layer was dried for isolating the desired pure product [Formula (I)] as brown solid. Yield; 130 mg (71%).

In another embodiment, the present invention provides a kit comprising of compound of formula (I) and polymeric/paper strip for the detection of SA (serum albumin) in urine.

In another embodiment, the present invention provides a compound of formula I used for detection of bio-thiols.

In yet another embodiment, the present invention provides use of compound of formula (I) for the detection of bio-thiols (cysteine and homocysteine).

The present invention provides a method for specific detection and quantification of bio-thiols using the compound of formula I.

BRIEF DESCRIPTION OF THE DRAWINGS:

Figure 1: Absorption spectra of Formula (I) (10 μM) (a) in absence and presence of 100 mole equivalent of different anions, cations and amino acids, (b) with varying [Cys] (0-100 mole equivalent), (c) with varying [Hcy] (0-100 mole equivalent) in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2).

Figure 2: Emission spectra of Formula (I) (10 μM) (a) in presence and absence of 100 mole equivalent of different amino acids, (b) with 0-100 equivalent of Cys (d) with 0-100 mole

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equivalent of Hcy. [inset: c, e shows change in intensity at 585 nm with concentration of Cys and Hcy respectively] in aq. HEPESbuffer:CH_3CN medium (7:3, v/v; pH 7.2) excited at 480 nm.

Figure 3: Interference study in presence of Formula (I) (10 μM) in presence of 100 mole equivalent of Cys and 200 mole equivalent of other aminoacids in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using $\lambda_{Ext}/\lambda_{Em}$: 480/585nm.

Figure 4: Interference study in presence of Formula (I) (10 μM) in presence of 100 mole equivalent of Hcy and 200 mole equivalent of other aminoacids in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using $\lambda_{Ext}/\lambda_{Em}$: 480/585nm.

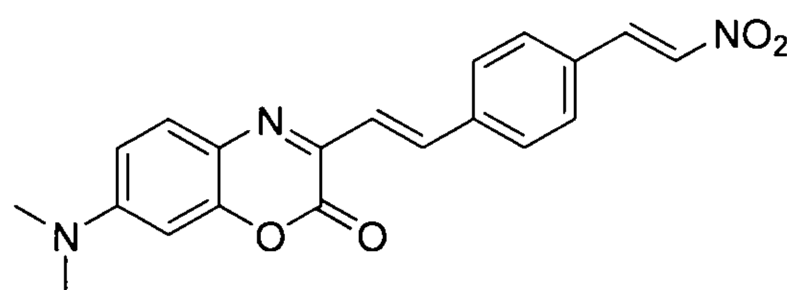
Figure 5: (a) Fluorescence spectra of probe L (5 μM) in presence of different concentration of HSA (0-20 μM). (b) Change in emission intensity in presence of different concentration of HSA. (c) Calibration plot and quantification of HSA in human urine.

Figure 6: Transition state Energy barrier and product stability of compound of Formula I (top) and compound disclosed in the prior art (Anila et al. already referred in the background of the invention) with Hcy at B3LYP/6-31+G*//B3LYP/6-31G* level of theory. (Distances are given in Å and energies are given in kcal/mol)

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail in connection with certain preferred and optional embodiments, so that various aspects thereof may be more fully understood and appreciated.

In the view of above, the present invention provides a compound of formula (I);



Formula I

In another embodiment, the present invention provides a process for the preparation of compound of formula (I) comprising the steps of:

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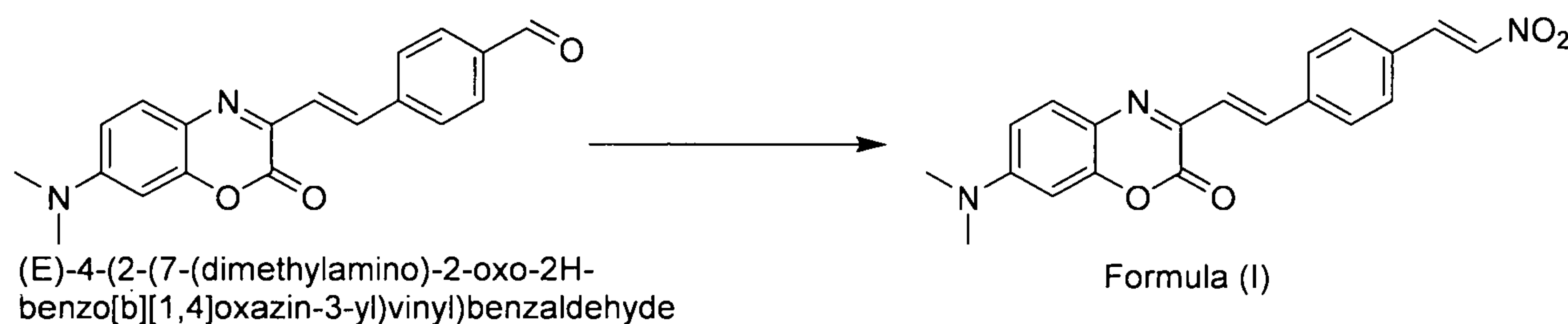
Step-1: To prepare Formula (I), 160 mg (0.5 mmol) of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde was dissolved in 10 ml of nitromethane. To it 385 mg (5 mmol) of ammonium acetate was added to it and heated at 80 °C for 4 h. On cooling to room temperature precipitate appeared, which was washed with water and then with 10 ml of diethylether. Residue was further extracted from DCM/water and the DCM layer was dried for isolating the desired pure product [Formula (I)] as brown solid. Yield; 130 mg (71%).

The process for the preparation of formula (I) is as shown scheme 1:



Scheme 1: Synthetic Methodology of Formula (I): NH_4OAc, CH_3NO_2, 80°C, 4h.

In yet another embodiment, the present invention provides use of compound of formula (I) for the detection of bio-thols (cysteine and homocysteine).

Still another objective of the present invention is to provide a kit for the detection of human serum albumin in urine comprising compound of formula (I).

Considering the possible applications in real sample and biology, the spectroscopic properties of Formula (I) were carried out in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2). The absorption spectrum of Formula (I) (10 μM) in an aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2) shows a strong band at 505 nm ($\varepsilon = 24100\ M^{-1}cm^{-1}$) and a weak band at 360 nm ($8200\ M^{-1}cm^{-1}$). The absorption spectrum of Formula (I) was recorded in absence and presence of 100 mole equivalent of different common anions (X: F^-,Cl^-, Br^-, $H_2PO_2^-$, OAc^-, HSO_4^-, CN^-), cations (Na^+, Ca^{2+}, Mg^{2+},Fe^{2+}, Fe^{3+}, Cu^{2+}, Cr^{3+}, Ni^{2+}, Zn^{2+}) and natural amino

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acids (AAs: histidine (His), leucine (Leu), methionine (Met), isoleucine(Ile), phenylalanine (Phe), tryptophon (Trp), tyrosine (Tyr), valine (Val), serine (Ser), alanine (Ala), arginine (Arg), glycine (Gly), glutamine (Gln), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), lysine (Lys), methionine (Met)), and biothiols like glutathione (GSH), Homocysteine (Hcy) and Cysteine (Cys) (Figure 1). A hypsochromic shift of ~25nm was observed for the 505 nm band only when spectra were recorded in presence of Cys and Hcy and this attributed to a visually detectable change in solution colour from red to orange. Other analytes failed to induce any detectable change in absorption spectra. Observed hypsochromic shift in absorption spectra supports the formation of Michael adducts with Cys and Hcy which interrupted the extended conjugation as well as disfavor the push-pull effect and the ICT process. Electronic spectra for the probe molecule Formula (I) (10 μM) were recorded for increasing concentration of Cys or Hcy (0.0 – 1.0 mM), which showed appearance of two new bands at 480 nm and 315 nm with concomitant decrease in band intensities at 505 nm and 360 nm (Figure 1b and 1c). Titration profile also revealed three isosbestic points at 285 nm, 330 nm, 400 nm which reflects reaction is proceeding without forming an intermediate.

Emission spectra of Formula (I) was recorded in presence and absence of 100 mole equivalents of different anions, cations and natural amino acids following excitation at 480 nm. Apparently a switch ON emission response with λ_{max} of 585 nm was observed for compound of Formula I only in presence of Cys, and Hcy (Figure 2). Other analytes including GSH did not show any detectable change in the emission spectra. The probe of Formula (I) shows a large stokes shift of ~105 nm, which was highly desirable as it would help in reduction of the self-absorption and background noise for improved fluorescence responses. Significant increase in fluorescence enhancement in presence of Cys and Hcy is

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attributed to the 1,4-addition that leads to the formation of L-Cys/Hcy adduct. Lack of extended conjugation in these adducts has contributed in achieving the enhanced HOMO-LUMO energy gap and this favors the radiative deactivation of the CT-based excited state, which results in the appreciable increase in emission quantum yield. Systematic changes in the emission spectra were recorded in HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2) with increasing [Cys] or [Hcy] (0 to 100 mole equivalent) and these spectra are shown in Figures 2a and 2b, respectively.

To validate the selectivity of Formula (I) for any practical application, interference studies were performed. Emission responses were recorded for Formula (I) (10 μM) after incubating with 100 mole equivalent of Cys (recorded after 5 min) or Hcy (recorded after 20 min in presence of excess (200 equivalent) of other amino acids. Even after one hour incubation of these resultant solutions, no further change in emission intensities was observed. This clearly nullified any interference other competing amino acids, including potential interfering agent GSH (Figure 3 and 4). These results indicate that the compound of Formula (I) can be used as a turn-on probe for selective detection of Cys/Hcy selectively even in presence other common interfering agents.

For practical application, the compound of Formula (I) was further utilized for detection of free Cys residue in proteins. We have employed Human Serum Albumin (HSA) as template protein because it is a major component of blood plasma and also as it contains only one free Cys residue (Cys 34). First, sensing of HSA was tested by addition of different concentration of protein with probe compound of Formula (I). As shown in figure 5, with increasing concentration of HSA from (0.5 μM to 20 μM), fluorescence intensity at 585 nm increase, with naked eye colour change from red to orange. This indicates that probe compound of

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Formula (I) reacts with the free Cys 34 residue of the protein. Next, we checked the time course of the reaction, and found it takes ~10 min to complete the reaction which is greater than the time taken for Cys. The pK_a value of Cys residue of HSA is 5.0 which is quiet low than the Cys/Hcy, so it is expected to react better than them. However, the slow reactivity of HSA can be attributed to the microenvironment of the protein residue which makes it difficult to undergo substitution reaction.

Considering above results, for real application the efficacy for the detection of HSA in biological system was determined. Healthy human urine contains less than 30 mg L^{-1} of HSA, but in kidney disease, HSA concentration in urine increases which cause protein urea. So HSA detection in urine can be useful in early detection of kidney disease and cardiovascular disease. Fresh urine sample from healthy donor was collected and diluted 10 times before use. Different concentration of HSA (0.5-20 μM) in HEPES buffer was prepared and then incubated with probe of Formula (I) (5 μM). Emission intensity at 585 nm shows a good linear relationship with HSA concentration and used as standard curve. Two known concentration of HSA (3 and 14 μM) were spiked with urine sample and then incubated with probe compound of Formula (I). Results from the calibration curve, seems to be in good agreement with high recovery rate using this method (Table 1). Thus, this provides a very simple method for detection of HSA in human urine.

Table 1. Determination of HSA in healthy human urine.

Sample no	HSA added (μM)	HSA found (μM)	Recovery (%)
1	3.0	2.81 ± 0.2	93.6
2	14.0	13.2 ± 0.3	94.2

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Formula (I) act as fluorescent turn-on probe for specific detection of biothiol in aqueous medium.

Moreover, the probe of Formula (I) can bind with free SH group of HSA with increase in emission. This has been used to detect HSA in human urine.

The following examples, which include preferred embodiments, will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purpose of illustrative discussion of preferred embodiments of the invention.

Examples Following examples are given by way of illustration therefore should not be construed to limit the scope of the invention.

Example 1

Synthesis of Formula (I):

(a) To prepare the probe of Formula (I), 160 mg (0.5 mmol) of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde was dissolved in 10 ml of nitromethane. To it 385 mg (5 mmol) of ammonium acetate was added to it and heated at 80 °C for 4 h. On cooling to room temperature precipitate appeared, which was washed with water and then with 10.0 ml of diethylether. Residue was further extracted from DCM/water and the DCM layer was dried for isolating the desired pure product as brown solid. Yield; 130 mg (71%)

(b) To prepare the probe of Formula (I), 160 mg (0.5 mmol) of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde was dissolved in 14.0 ml of nitromethane. To it 385 mg (5 mmol) of ammonium acetate was added to it and heated at 80 °C for 4 h. On cooling to room temperature precipitate appeared, which was washed with water and then with 10 ml of diethylether. Residue was further extracted from DCM/water and the DCM layer was dried for isolating the desired pure product as brown solid. Yield; 126 mg (68%)

(c) To prepare the probe of Formula (I), 160 mg (0.5 mmol) of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde was dissolved in 10.0 ml of nitromethane. To it 385 mg (5 mmol) of ammonium acetate was added to it and heated at 80 °C for 4 h. On cooling to room temperature

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precipitate appeared, which was washed with water and then with 10 ml of diethylether. Residue was further extracted from chloroform/water or ethyl acetate/water and the chloroform or ethyl acetate layer was dried for isolating the desired pure product as brown solid. Yield; 69% (for chloroform) and 70% (for ethyl acetate). (I have extracted only from DCM and Water)

^{1}H NMR (400 MHz, $CDCl_3$) δ:8.27 (d, J = 13.2Hz, 1H), 8.14 (d, J = 13.6Hz, 1H), 7.88 (m, 2H), 7.81 (m, 2H), 7.56 (d, J=9.2Hz, 1H), 7.50 (d, J=16Hz, 1H), 6.83 (dd, J=2.4, 8.8 Hz, 1H), 6.61 (d, J=2.4, 1H), 3.08 (s, 3H). ^{13}C NMR (400 MHz, CPMAS, 8 kHz) δ: 153.1, 151.2, 147.9, 140.5, 136.4, 130.1, 127.3, 124.1, 109.6, 94.5, 39.53. IR ν_{max}/cm^{-1}: 2914, 1728, 1616, 1467. HRMS (m/z): Calculated $[C_{20}H_{17}N_3O_4+H]^+$ 364.1292 and found 364.1285.

Example 2: Detection of bio-thiols by Kit

General experimental procedure for UV-Vis and Fluorescence studies:

1.0 mM stock solution of Formula (I) acetonitrile was prepared and used for further studies after appropriate dilution using 10 mM HEPES buffer achieving the effective concentration of 1.0×10^{-5} M in 10 mM aq. HEPES buffer-CH_3CN (7:3, v/v; pH 7.2). Stock solutions of 100 mM of different amino acids were prepared in a 10 mM aq. HEPES buffer-CH_3CN (7:3, v/v; pH 7.2) medium. Stock solutions were further diluted with buffer solution as per requirement for a specific experiment.

Example 3: Comparison of Transition state Energy barrier and product stability of compound of Formula I and prior art compound (Anila et al. already referred in the background of the invention) with Hcy at B3LYP/6-31+G*//B3LYP/6-31G* level of theory. (Distances are given in Å and energies are given in kcal/mol)

B3LYP/6-31+G*//B3LYP/6-31G*: From the molecular mechanics conformational search, the conformations with relative energies (*E*rel, calculated energies relative to the lowest energy conformation) ≤ 5 kcal/mol were investigated using density functional theory (DFT) employing the popular hybrid B3LYP functional and the 6–31G* basis set, M06 is combination functional and the 6–31G* basis set. The B3LYP functional is popular for modeling organic compounds, the M06 functional was chosen because it was developed to

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predict accurate structures and energies of main-group-containing compounds and includes concovalent interactions. The 6–31G* basis set was chosen for its relatively rapid calculations. In order to confirm the energies from the B3LYP and M06 analyses, single-point Hartree-Fock (HF), followed by second-order Møller-Plesset electron correlation (MP2) calculations at the 6–31G** level were carried out using the B3LYP geometries.

The structures of cysteine, homocysteine and compound of Formula I were fully optimized with density functional theory (DFT) and standard 6-31G* basis set. Frequency calculations were performed at the same level of theory, to conform that each stationary points a local minimum (zero imaginary frequency) or transition states (one imaginary frequency). All the calculations have been performed with GAUSSIAN 09suite of program. Quadratic Synchronous Transit (QST2) method was employed to locate transition structures.

A comparison of Transition state Energy barrier and product stability of compound of Formula I and prior art compound (Anila et al. already referred in the background of the invention) is provided in Figure 6. The transition states have been calculated with B3LYP/6-31G* level of theory. In the transition state geometry, the distance between the 'C' atom of compound of Formula I / prior art compound and 'S' atom of Hcy was found to be ~2.6Å. The single point calculations were performed with B3LYP/6-31+G* level of theory in aqueous phase using B3LYP/6-31G* geometry to examine the activation barrier for these reactions. T

The products formed by reacting compound of Formula I and the prior art compound with Hcy have been optimized at B3LYP/6-31G* level of theory. The B3LYP/6-31+G*//B3LYP/6-31G* calculated reaction energies for product suggest that compound of Formula I with Hcy is thermodynamically more stable than that of the prior art compound by 1.3kcal/mol. The calculated reaction energies at different levels of theory also showed the

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similar trend as observed with B3LYP/6-31+G*//B3LYP/6-31G* level of theory. The sluggishness observed for the prior art compound with Hcy may arise due to the lesser affinity of the prior art compound towards Hcy (Figure 6). The calculated mulliken charges show that the charges on C1 is relatively more negative in the prior art compound as compared to compound of Formula I and hence the nucleophile Hcy will experience more repulsion while interacting with the C1 carbon of the former ligand. These computational results qualitatively corroborate the experimental findings and suggest the sluggishness in the reaction of prior art compound with Hcy. This clearly provide how nitroolefin based benzooxazine derivative differ in their mode of action from Nitroolefin-based coumarin compounds in specifically detecting Hcy.

Advantages of the invention:

1. The reagent comprising the compound of Formula I used in the present invention is a fluorescent turn-on probe for specific detection of biothiol in physiological condition. This probe displays an emission enhancement on reaction with thiols, which break the conjugation between nitroolefin and benzooxazine group.
2. This probe can bind with free SH group of HSA with increase in emission.
3. This probe can also be used to detect HSA in human urine.
4. This probe can also be used for the quantitative detection of cysteine and homocysteine.
5. The present invention further relates to a kit comprising compound of formula (I).

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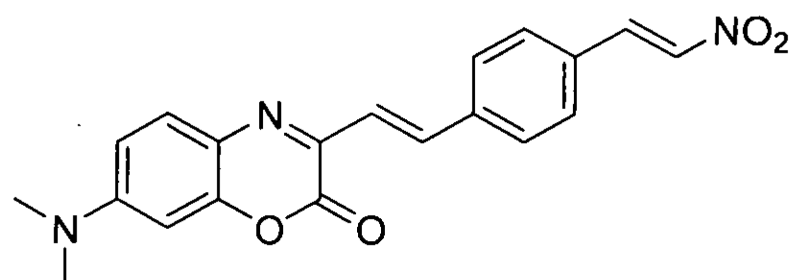
We Claim:

1. A compound of formula I

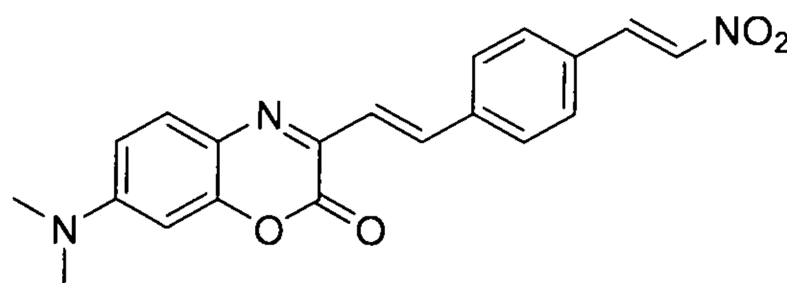


Formula I

2. The compound as claimed in claim 1, used for the detection of cystine and homocystine.

3. The compound as claimed in claim 1, used to bind free SH group (thiol group) of SA (serum albumin) with increase in emission which detect SA in urine.

4. A process for the preparation of compound of formula I comprising the steps of:



Formula I

i. dissolving (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl) benzaldehyde in nitromethane at a temperature in the range of 50-100°C;

ii. adding ammonium acetate to the solution obtained in step (i) with stirring for 3-9 hours;

iii. cooling the reaction mixture obtained in step (ii) to room temperature;

iv. washing the solid obtained in step (iii) to obtain the residue;

v. extracting the residue obtained in step (iv) with water and organic solvent and

vi. drying out the organic solvent layer to obtain the compound of formula (I).

5. The process as claimed in claim 4, wherein the ratio of (E)-4-(2-(7-(dimethylamino)-2-oxo-2H-benzo[b][1,4]oxazin-3-yl)vinyl)benzaldehyde and nitromethane is in the range of 1:71 to 1:100.

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6. The process as claimed in claim 4, wherein the washing is performed using water and diethyl ether.

7. The process as claimed in claim 4, wherein the organic solvent is selected from the group consisting of dicholoromethane, chloroform and ethyl acetate.

8. A kit comprising of compound of formula (I) and polymeric/paper strip for the detection of SA (serum albumin) in urine.

9. The compound of formula I as and when used for detection of bio-thiols.

10. A method for specific detection and quantification of bio-thiols using the compound of formula I.

Dated this 9th day of February 2018

Scientist

Innovation Protection Unit

Council of Scientific and Industrial Research

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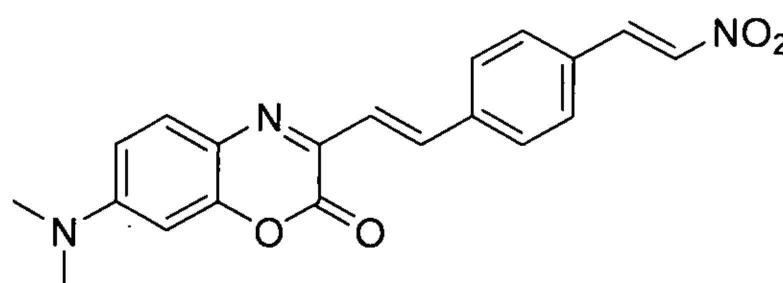
A COMPOUND FOR THE DETECTION OF BIO-THIOLS AND PROCESS FOR PREPARATION THEREOF

Abstract

The thio-biomolecules which play crucial roles in biological systems are cysteine, homocysteine and glutathione. The present invention relates to compound of formula I which can detect bio-thiols (cysteine and homocysteine) in aqueous medium. The compound of formula I is a sensor molecule for fluorescence or luminescence-based detection of cysteine and homocysteine analyte. More particularly, the present invention relates to a compound of formula (I), process for preparation thereof and use of compound of formula (I) for detecting bio-thiols. The present invention further relates to a kit comprising compound of formula (I). A kit is prepared by using the compound of formula I for the detection of cysteine and homocysteine.



Formula I

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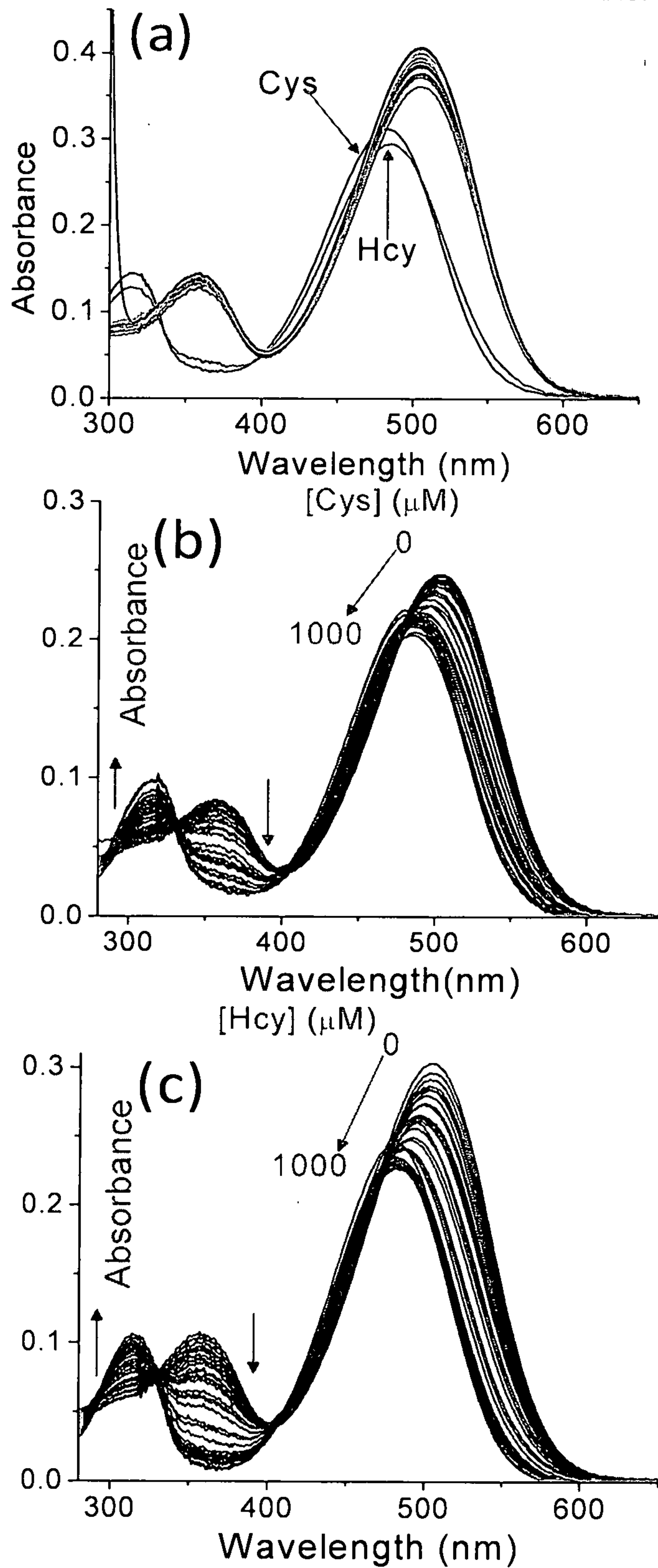

Figure 1

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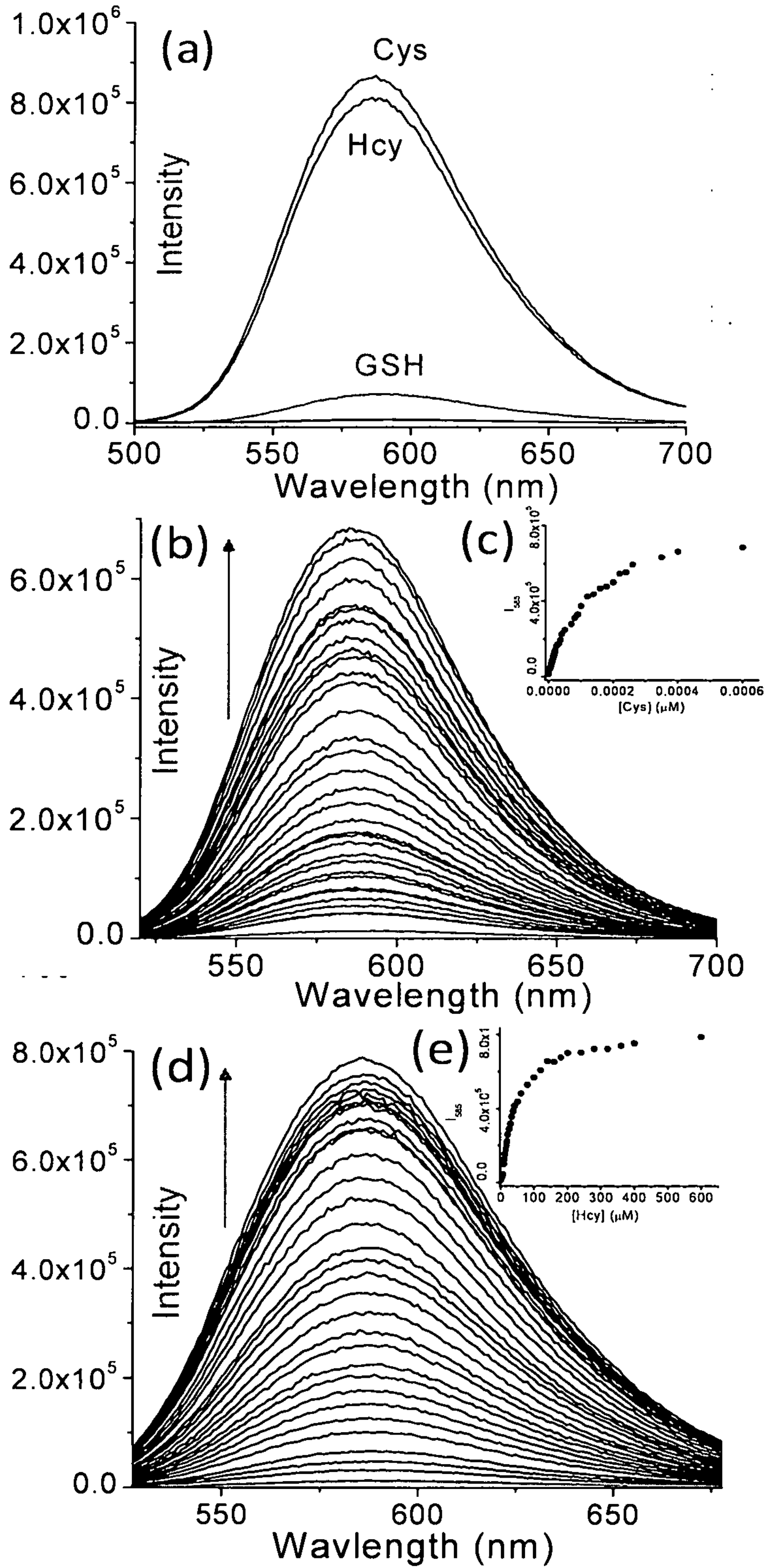

Figure 2

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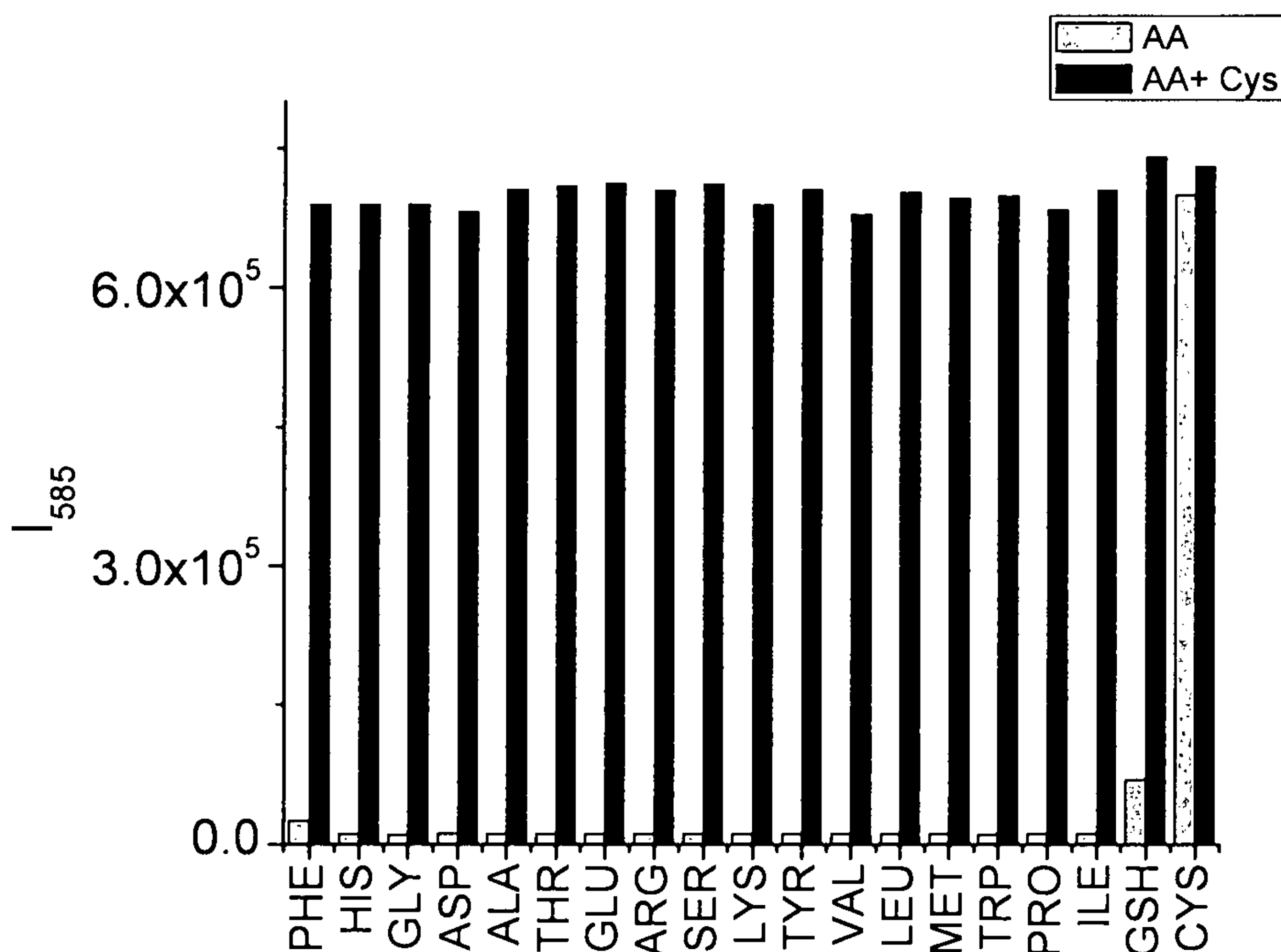

Figure 3

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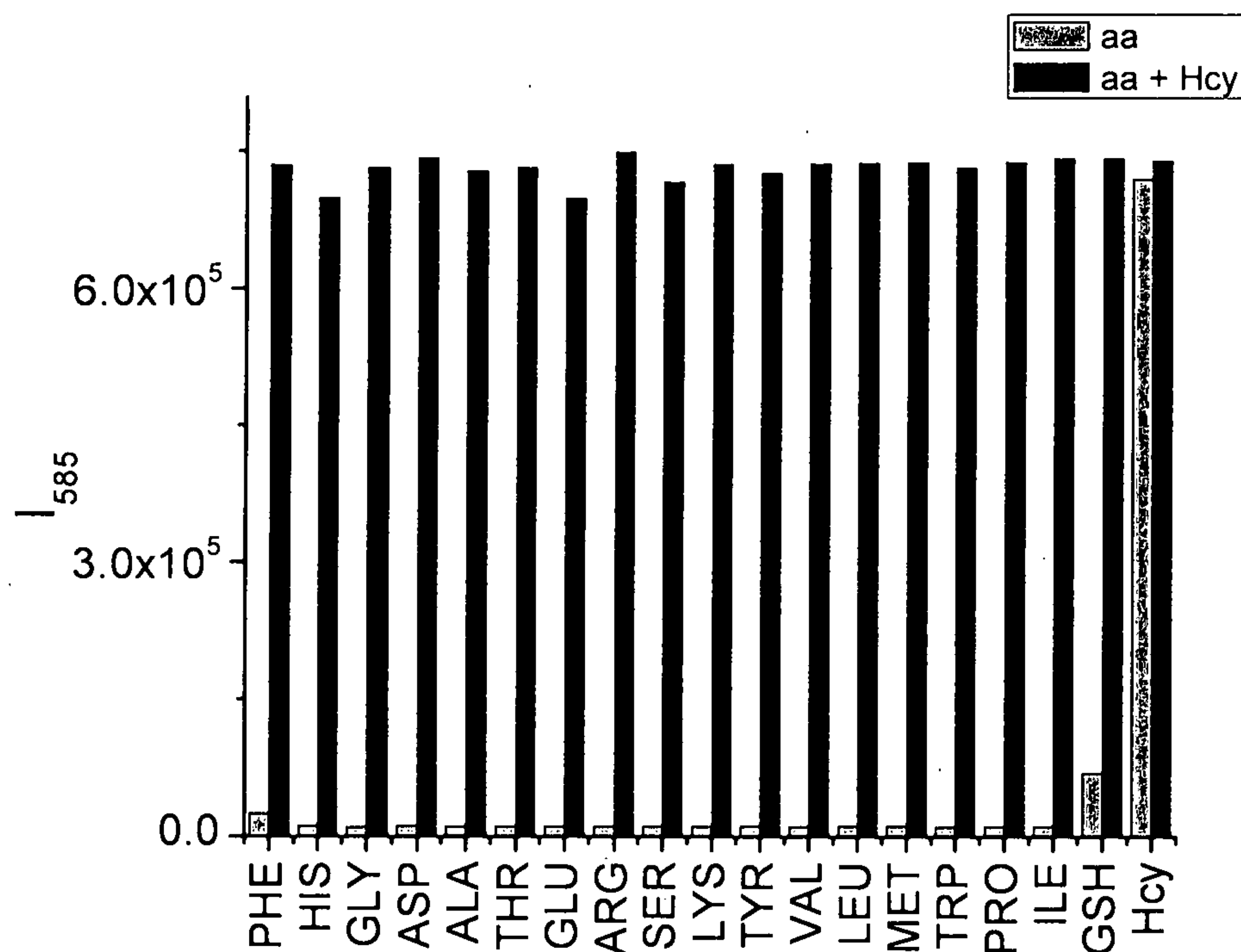

Figure 4

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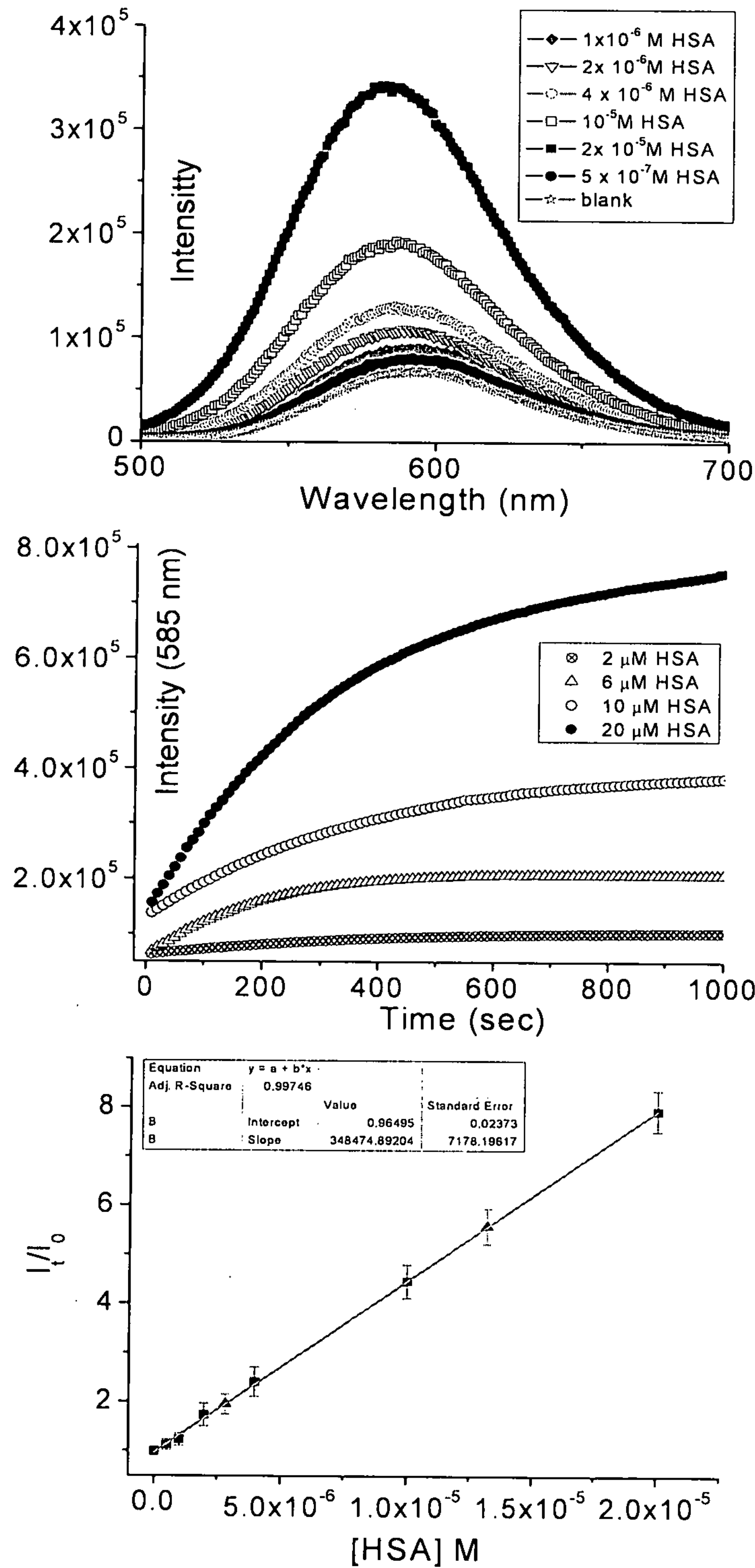

Figure 5

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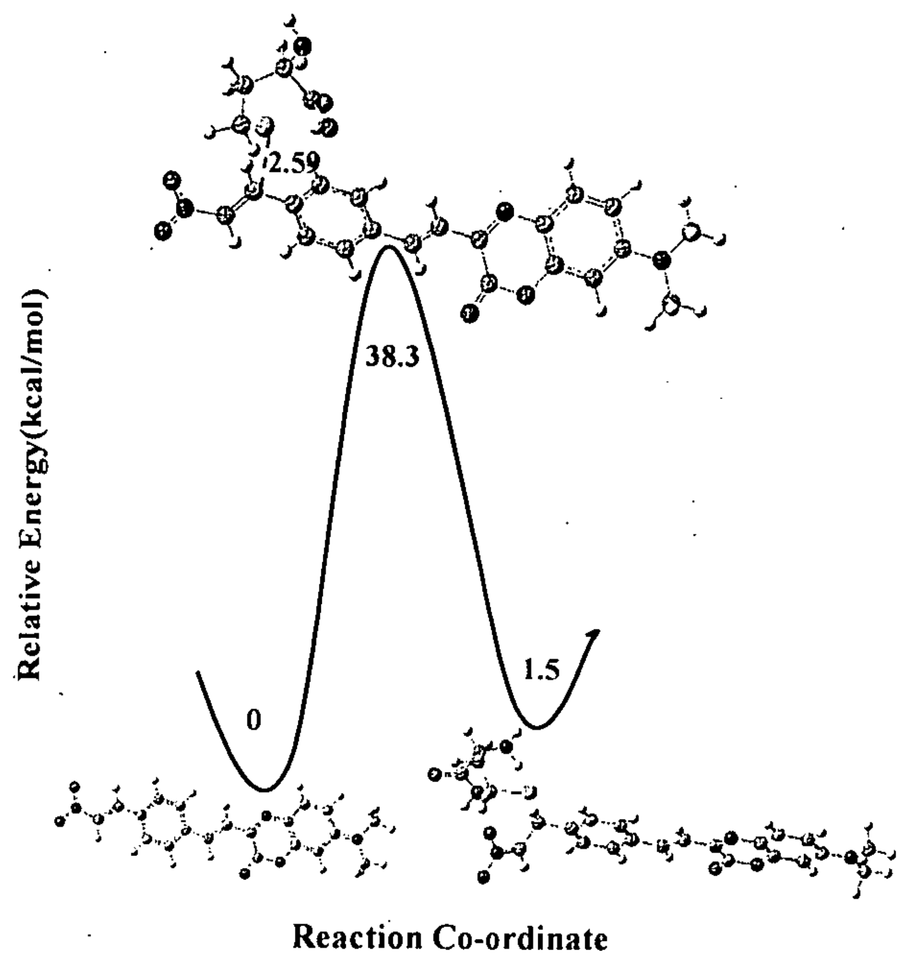
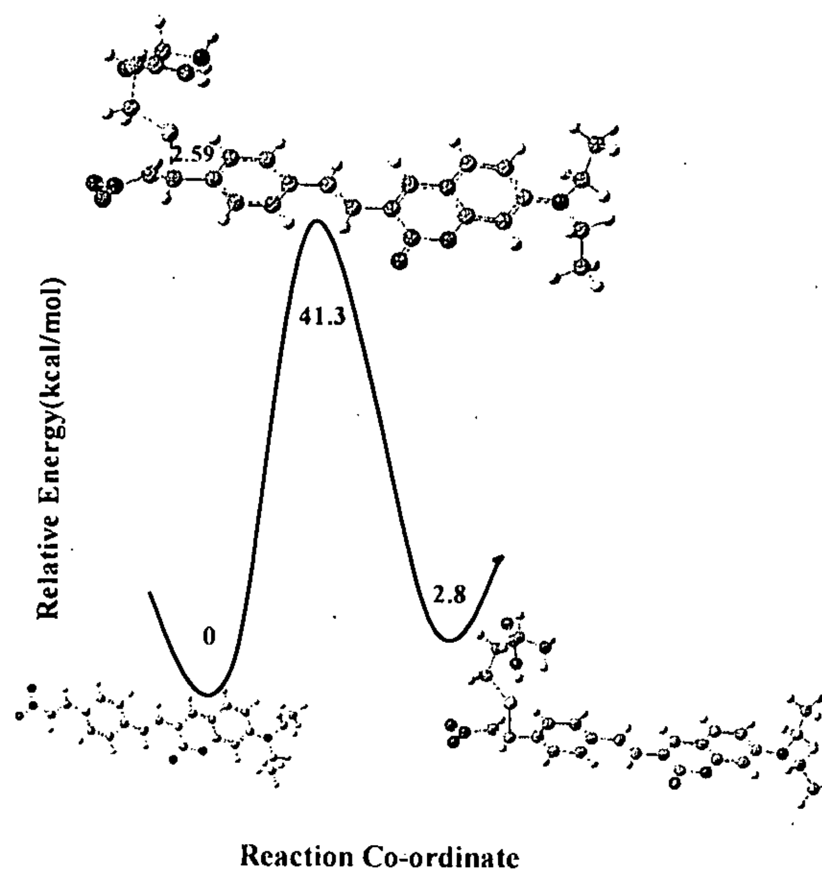


Formula I



Prior art compound

Figure 6

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