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(54) Title: COMPOSITIONS AND METHODS FOR ANALYZING CYSTEINE

(57) Abstract: The present invention relates to compositions and methods for determining the absolute configuration of D/L-cysteine and/or the enantiomeric composition of cysteine and/or the concentration of total cysteine in a sample. Uses of the composition and method are also described.

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COMPOSITIONS AND METHODS FOR ANALYZING CYSTEINE

[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 62/489,411, filed April 24, 2017, which is hereby incorporated by reference in its entirety.

- 5 [0002] This invention was made with government support under grant numbers CHE-1213019 and CHE-1464547 awarded by the National Science Foundation. The government has certain rights in this invention.

FIELD OF THE INVENTION

- 10 [0003] The present technology relates to probes and methods for evaluating the absolute configuration, and/or enantiomeric composition, and/or overall concentration of free cysteine in a sample.

BACKGROUND OF THE INVENTION

- [0004] Amino acids are inarguably among the most predominant and important chiral compounds in nature. For a long time, it has been assumed that L- amino acids are almost exclusively present in higher animals while the corresponding D-enantiomers would only be utilized by microorganisms and bacteria. In recent years, this view of homochirality has been revisited as substantial amounts of D-amino acids have been detected in mammals and humans. In fact, D- and L-amino acids are not mutually exclusive and often co-exist as nonracemic mixtures. This is the case in mammalian and human tissue where they occur in the free form or in proteins as well as in the central nervous system (CNS). (Hamase et al., *J. Chromatogr. B* 781:73–91 (2002); Walsh, *ACS Chem. Biol.* 9:1653–1661 (2014).) The total amount and enantiomeric excess or ratio (ee or er) of amino acids in the CNS play an important role in human physiology and pathology and they have been associated with neurological disorders such as schizophrenia, Parkinson's, Huntington's, and Alzheimer's disease and other neurodegenerative or psychiatric disorders such as traumatic stress and anxiety attacks. (Fuchs et al., *Mol. Genet. Metab.* 85:168–180 (2005).) The widespread occurrence, racemization processes, and pivotal biological functions of D-amino acids have important implications in the life sciences. (Friedman, *Chem. Biodivers.* 7:1491–1530 (2010); D-AMINO ACIDS IN
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CHEMISTRY, LIFE SCIENCES, AND BIOTECHNOLOGY (Hans Bruckner & Noriko Fujii eds., 2011); Friedman & Levin, *Amino Acids* 42:1553–1582 (2012); Simon et al., *J. Am. Chem. Soc.* 138: 12099–12111 (2016).) The ability to quantify both concentration and enantiomeric composition of amino acids with artificial receptors is considered very promising for the study, diagnosis, and treatment of diseases and ageing. (Turner, *Chem. Soc. Rev.* 42:3184–3196 (2013).) Although increasing efforts have recently been directed to optical chemosensing, a robust method that accurately reports the total amount and ee/er at the micromolar concentration level of a specific amino acid in the presence of others is still elusive. (Leung et al., *Chem. Soc. Rev.* 41:448–479 (2012); Wolf & Bentley, *Chem. Soc. Rev.* 42:5408–5424 (2013); Wu et al., *Chem. Rev.* 115:7893–7943 (2015); Zhou & Yoon, *Chem. Soc. Rev.* 41:52–67 (2012).)

[0005] The change in the dogmatic perception of mammalian amino acid homochirality has shifted increasing attention to the natural occurrence and the distinct biological, nutritional, and medicinal roles of the D-enantiomer of cysteine and of D/L-enantiomeric mixtures in recent years. (Sit et al., *Acc. Chem. Res.* 44: 261–268 (2011); Kimura, *Nitric Oxide* 41:4–10 (2014); Yuan & Liang, *Org. Biomol. Chem.* 12:865–871 (2014); Shibuya et al., *Nat. Commun.* 4:1366 (2013).) These findings underscore the need for a means that permits fast determination of the absolute configuration, enantiomeric composition (ee and/or er), and overall concentration of cysteine in aqueous mixtures. Selective stereochemical cysteine analysis, however, is particularly complicated because of the common presence of chemically similar biothiols. In addition to other amino acids, homocysteine (Hcy) and glutathione (GSH) typically interfere with molecular recognition processes and further complicate both substrate-specific detection and enantioselective quantification. Significant progress with regard to chemosensing of biothiols with molecular UV or fluorescent probes has been reported over the past decade. (Chen et al., *Chem. Soc. Rev.* 39:2120–2135 (2010); Lee et al., *Chem. Soc. Rev.* 44:4185–4191 (2015); Kubota & Hamachi, *Chem. Soc. Rev.* 44: 4454–4471 (2015); Kowada et al., *Chem. Soc. Rev.* 44:4953–4972 (2015); Niu et al., *Chem. Soc. Rev.* 44:6143–6160 (2015).) A variety of fluorescence, colorimetric/UV, and electrochemical assays for selective quantification of Cys, Hcy, or GSH have been introduced. (Maeda et al., *Angew. Chem. Int. Ed.* 44:2922–2925 (2005); Bouffard et al., *Org. Lett.* 10:37–40

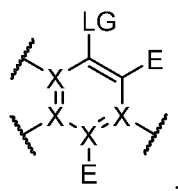
(2008); Zhang et al., *Org. Lett.* 11:1257–1260 (2009); Lin et al., *Chem. Eur. J.* 15:5096–5103 (2009); Li et al., *Chem. Commun.* 5904–5906 (2009); Liu et al., *J. Am. Chem. Soc.* 136:574–577 (2014); Wang et al., *Chem. Commun.* 52:827–830 (2016); Shen et al., *Anal. Methods* 8:2420–2426 (2016); Tang et al., *RSC Adv.* 6:34996–35000 (2016).) (For selective thiophenol sensing, see Jiang et al., *Angew. Chem. Int. Ed.* 46:8445–8448 (2007); Han & Kim, *Tetrahedron* 60:11251–11257 (2004); Huo et al., *Org. Lett.* 11:4918–4921 (2009); Dai et al., *Anal. Chim. Acta* 900:103–110 (2015); and Luo et al., *Angew. Chem. Int. Ed.* 54:14053–14056 (2015).) While some of these methods can be used for quantitative analysis of cysteine in aqueous solutions in the presence of Hcy and GSH, additional information about the enantiomeric composition cannot be obtained. The use of silver nanoparticles for the detection of cysteine enantiomers has shown promise but does not achieve combined concentration and ee analysis. (Zhang & Ye, *Anal. Chem.* 83:1504–1509 (2011).)

[0006] Therefore, there is still a need for a practical chiroptical sensing assay that is compatible with low cysteine concentrations (*e.g.*, micromolar) and allows rapid determination of the absolute configuration, enantiomeric composition, and overall concentration of free-form cysteine.

[0007] The present technology is directed to overcoming these and other deficiencies in the art.

SUMMARY OF THE INVENTION

[0008] One aspect of the present technology relates to a compound comprising a chromophore of the formula

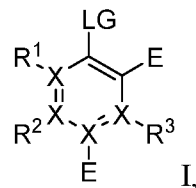


wherein:

- each X is independently C or N;
- each E is independently hydrogen or an electron withdrawing group selected from the group consisting of $-\text{CF}_3$, $-\text{C}(\text{O})-\text{R}_a$, $-\text{SO}_2-\text{R}_a$, $-\text{CN}$, and $-\text{NO}_2$, with the proviso that at least one E is an electron withdrawing group; and
- LG is a leaving group.

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[0009] In at least one embodiment of this aspect of the present technology, the compound is a compound of Formula I:



wherein:

- 5 each X is independently C or N;
- each E is independently hydrogen or an electron withdrawing group selected from the group consisting of $-\text{CF}_3$, $-\text{C}(\text{O})-\text{R}_a$, $-\text{SO}_2-\text{R}_a$, $-\text{CN}$, and $-\text{NO}_2$, with the proviso that at least one E is an electron withdrawing group;
- LG is a leaving group;
- 10 R^1 and R^2 are each independently absent or selected from the group consisting of $-\text{R}_a$, $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; or R_1 and R_2 , together with the carbon atoms to which they are attached, form a cycloalkyl, heterocycloalkyl, aryl, or heteroaryl;
- R^3 is absent or selected from the group consisting of $-\text{R}_a$, $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; and
- 15 each R_a is independently selected from the group consisting of $-\text{H}$, $-\text{alkyl}$, $-\text{O-alkyl}$, $-\text{N-alkyl}$, $-\text{alkenyl}$, $-\text{O-alkenyl}$, $-\text{N-alkenyl}$, $-\text{alkynyl}$, $-\text{O-alkynyl}$, $-\text{N-alkynyl}$, $-\text{aryl}$, $-\text{O-aryl}$, $-\text{N-aryl}$, $-\text{heteroaryl}$, $-\text{O-heteroaryl}$, $-\text{N-heteroaryl}$, $-\text{cycloalkyl}$, $-\text{O-cycloalkyl}$, $-\text{N-cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{O-heterocycloalkyl}$, and $-\text{N-heterocycloalkyl}$.
- 20

[0010] Another aspect of the present technology relates to an analytical method comprising:

- contacting a sample potentially containing D-cysteine, L-cysteine, or a mixture thereof with a probe according to the present technology, wherein said
- 25 contacting is carried out under conditions effective to result in double *ipso*-substitution of any cysteine present in the sample; and
- determining, based on any double *ipso*-substituted cysteine that forms, the absolute configuration of D/L-cysteine in the sample, and/or the enantiomeric composition of cysteine in the sample, and/or the total concentration of
- 30 cysteine in the sample.

[0011] Described herein are probes that carry a transferable aryl/heteroaryl moiety for substrate-specific optical analysis of cysteine. These probes provide smooth *N,S*-di(hetero)arylation of cysteine via double *ipso*-substitution and concomitant (chir)optical (*e.g.*, UV and circular dichroism) sensor readouts at high wavelengths. The absolute configuration of the major cysteine enantiomer and the enantiomeric composition (*i.e.*, enantiomeric excess and/or enantiomeric ratio) of D/L-cysteine can be determined from the sign and amplitude, respectively, of the induced CD signals, and a new UV absorption band correlates to the overall cysteine concentration. The methods described herein use a practical mix-and-measure protocol and fast UV (or other colorimetric) and CD (or other optical) measurements that provide accurate stereochemical information with samples covering a wide concentration range and drastically different D/L-cysteine ratios in, *e.g.*, body fluids. The presence of other amino acids and biothiols does not interfere with the cysteine sensing.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figures 1A–B relate to chiroptical cysteine sensing. Figure 1A shows the structures of probes **1–3** (top) and a general scheme (bottom) of double *ipso*-substitution for chiroptical cysteine sensing using probe **1** as an example. Figure 1B is CD spectra showing circular dichroism responses to the D and L enantiomers of cysteine using probe **1** (left) and probe **3** (right). The CD measurements were taken at 0.11 mM in acetonitrile:water (4:1).

[0013] Figures 2A–C relate to chiroptical cysteine sensing and reaction analysis. Figure 2A is the UV (left) and CD (right) spectra of Cys samples of varying concentration and ee using two equivalents of probe **1**. Figure 2B shows the NMR analysis of the formation of diarylated cysteine **4**. All NMR spectra were collected using deuterated acetonitrile:water (4:1) as solvent and K₂CO₃ as base. (1) Reference spectrum of diarylated cysteine **4**. (2) Potassium phenolate. (3) Cysteine sensing experiment after 7 minutes. (4) Probe **1**. (5) Cysteine. Figure 2C is the CD spectra of CD sensing of L-cysteine using probe **1** and different base additives showing the effect of the base on the CD response. (At 425nm, the lines correspond, from top to bottom, to LiOH, Na₂CO₃, CsOH, NaOH, DBU, TBAOH, K₂CO₃, and KOH.) The

CD and UV measurements were taken in acetonitrile:water (4:1) at 50 and 30 μ M, respectively.

[0014] Figures 3A–C relate to the determination of cysteine specificity. Figure 3A shows the structures of the amino acids and biothiols tested. Figure 3B is the CD (left) and UV (right) spectra showing sensing of L-Cys using probe **1** in the presence of the L-enantiomers of Phe, Ala, Ser, and Tyr. Figure 3C is the CD (left) and UV (right) spectra showing substrate specificity of probe **1** in the presence of homocysteine (HCy) and glutathione (GSH). The CD and UV measurements were taken in acetonitrile:water (4:1) at 110 and 30 μ M, respectively.

[0015] Figures 4A–B relate to quantitative cysteine recognition. Figure 4A is the CD spectra (left) showing the CD response of probe **1** to L-cysteine at varying concentrations and a plot (right) of the CD amplitudes at 376 nm (positive Y-axis) and 423 nm (negative Y-axis) of the sensing adduct versus cysteine concentration. The peak amplitudes are proportional to the cysteine concentrations tested, which shows the linearity of ee sensing vs L-Cys sample concentration. Figure 4B is the CD spectra (left) showing quantitative chiroptical sensing of L-cysteine and D-cysteine at 15 μ M concentration and a plot (right) showing the linear relationship between the CD effects at 377 nm (lower left and upper right quadrants) and 417 nm (upper left and lower right quadrants) versus enantiomeric excess (% ee) of cysteine. (See Figure 34 for the CD spectra of each ee tested.)

[0016] Figures 5A–B are the ^1H NMR (Figure 5A) and ^{13}C NMR (Figure 5B) spectra of probe **1** in CDCl_3 .

[0017] Figures 6A–B are the ^1H NMR (Figure 6A) and ^{13}C NMR (Figure 6B) spectra of probe **2** in CDCl_3 .

[0018] Figures 7A–B are the ^1H NMR (Figure 7A) and ^{13}C NMR (Figure 7B) spectra of probe **3** in CDCl_3 .

[0019] Figures 8A–B are the ^1H NMR (Figure 8A) and ^{13}C NMR (Figure 8B) spectra of *N,S*-bis(2,4-dinitrophenyl)cysteine in $(\text{CD}_3)_2\text{CO}$.

[0020] Figure 9 is the COSY spectrum of *N,S*-bis(2,4-dinitrophenyl)cysteine in $(\text{CD}_3)_2\text{CO}$.

[0021] Figure 10 is the HSQC spectrum of *N,S*-bis(2,4-dinitrophenyl)cysteine in $(\text{CD}_3)_2\text{CO}$.

- [0022] Figure 11 is the HMBC spectrum of *N,S*-bis(2,4-dinitrophenyl)cysteine in (CD₃)₂CO.
- [0023] Figure 12 is the DEPT spectrum of *N,S*-bis(2,4-dinitrophenyl)cysteine in (CD₃)₂CO.
- 5 [0024] Figure 13 is the NOESY spectrum of *N,S*-bis(2,4-dinitrophenyl)cysteine in (CD₃)₂CO.
- [0025] Figure 14 shows the crystal structure of probe 1.
- [0026] Figure 15 shows the crystal structure of probe 2.
- [0027] Figure 16 shows the crystal structure of probe 3.
- 10 [0028] Figure 17 is the CD spectra obtained using probe 1 with L-cysteine and D-cysteine. CD measurements were taken at 110 μM in acetonitrile.
- [0029] Figure 18 is the CD spectra obtained using probe 2 with L-cysteine and D-cysteine. CD measurements were taken at 110 μM in acetonitrile.
- [0030] Figure 19 is the CD spectra obtained using probe 3 with L-cysteine and
- 15 D-cysteine. CD measurements were taken at 110 μM in acetonitrile.
- [0031] Figure 20 shows a comparison of the CD spectra obtained with L-cysteine and probes 1–3. All CD measurements were taken at 110 μM in acetonitrile.
- [0032] Figure 21 is the CD spectra showing CD sensing of L-cysteine with probe 1 in different solvents.
- 20 [0033] Figure 22 is the UV spectra showing the comparison of L-cysteine UV sensing with probe 1 in different solvents.
- [0034] Figure 23 is the CD spectra showing sensing of L-cysteine in the presence of varying amounts of K₂CO₃.
- [0035] Figures 24A–B relate to the reaction between cysteine and probe 1 over
- 25 time. Figure 24A is the UV spectra showing the UV analysis of the reaction between cysteine and probe 1 at different time points. Figure 24B is a plot of the absorbance of probe 1 at 355 nm versus time.
- [0036] Figures 25A–B are the CD spectra (Figure 25A) and UV spectra (Figure 25B) obtained of reaction mixtures with a 1:1 or 2:1 probe:cysteine ratio. CD
- 30 measurements were taken at 140 μM in acetonitrile. UV measurements were taken at 30 μM in acetonitrile. (Reaction mixtures: 1 equivalent (“1:1”) or 2 equivalents (“2:1”) of probe 1 with L-cysteine and K₂CO₃ in CH₃CN:water (4:1).)

[0037] Figure 26 is the CD spectra showing sensing of synthesized *N,S*-bis(2,4-dinitrophenyl)cysteine from probe 1 and L-cysteine. CD measurements were taken at 240 μ M in acetonitrile.

[0038] Figure 27 is the ^1H NMR spectra relating to the analysis of probe 1 and L-cysteine. (1) *N,S*-bis(2,4-dinitrophenyl)cysteine; isolated product of the reaction between L-cysteine and 2 equivalents of probe 1 (in the presence of 4 equivalents of K_2CO_3); (2) Phenol in the presence of 4 equivalents of K_2CO_3 ; (3) Reaction mixture of L-cysteine with 2 equivalents of probe 1 in the presence of 4 equivalents of K_2CO_3 , after 7 minutes; (4) Probe 1 in the presence 4 equivalents of K_2CO_3 ; (5) L-cysteine in the presence of 4 equivalents of K_2CO_3 .

[0039] Figure 28 is the CD spectra of the sensing mixture at 0, 10, 30, 60, and 120 minutes.

[0040] Figure 29 is the ^1H NMR spectra showing continued ^1H NMR analysis of the sensing mixture.

[0041] Figures 30A–B are the UV spectra (Figure 30A) showing the UV response of probe 1 measured over a cysteine concentration range from 100 to 1000 μ M and a plot (Figure 30B) of the UV absorbance at 355 nm.

[0042] Figure 31 is a picture of various solutions showing the naked eye detection of cysteine at 1.25 mM. A: L-Serine + probe 1; B: L-Phenyl Alanine + probe 1; C: L-Alanine + probe 1; D: L-Tyrosine + probe 1; E: L-Cysteine + probe 1; F: Probe 1.

[0043] Figures 32A–B are the UV spectra (Figure 32A) obtained from the reaction between probe 1 and varying amounts of L-cysteine and a plot (Figure 32B) of the UV absorbance at 355 nm versus the concentration of L-cysteine. The absorbance values are proportional to the cysteine concentrations tested.

[0044] Figures 33A–B are the CD spectra (Figure 33A) showing the chiroptical response of probe 1 to scalemic samples of L-cysteine and a plot (Figure 33B) of the CD amplitudes at 375 nm (lower left and upper right quadrants) and 422 nm (upper left and lower right quadrants) versus sample ee. The peak amplitudes are proportional to the enantiomeric excess of the sample.

[0045] Figure 34 is the CD spectra showing the chiroptical response of probe 1 to scalemic samples of cysteine at 15 μ M. The peak amplitudes are proportional to the enantiomeric excess of the sample. (See also Figure 4B.)

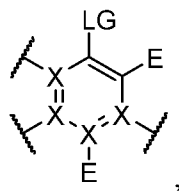
[0046] Figures 35A–B are the UV spectra (Figure 35A) obtained from the reaction between probe 1 and varying concentrations of L-cysteine and a plot (Figure 35B) of the UV absorbance measured at 355 nm versus the concentration of L-cysteine. The absorbance values are proportional to the cysteine concentrations tested.

[0047] Figures 36A–B are the CD spectra (Figure 36A) showing the chiroptical response of probe 1 to scalemic samples of cysteine and a plot (Figure 36B) of the chiroptical response at 365.5 nm (lower left and upper right quadrants) and 409 nm (upper left and lower right quadrants) versus cysteine ee. The peak amplitudes are proportional to the enantiomeric excess of the sample.

DETAILED DESCRIPTION

[0048] The present technology relates to compositions and methods related to a practical chiroptical sensing assay that is compatible with aqueous solutions (*e.g.*, body fluids) and allows rapid determination of the absolute configuration, enantiomeric composition, and overall concentration of cysteine at low (*e.g.*, micromolar) concentrations. The methods are highly specific for free-form cysteine, even in the presence of agents that interfere with known molecular recognition processes, such as other amino acids and biothiols (*e.g.*, homocysteine and glutathione).

[0049] One aspect of the present technology relates to a compound comprising a chromophore of the formula



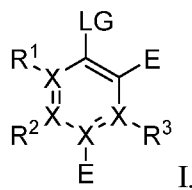
wherein:

each X is independently C or N;

each E is independently hydrogen or an electron withdrawing group selected from the group consisting of $-\text{CF}_3$, $-\text{C}(\text{O})-\text{R}_a$, $-\text{SO}_2-\text{R}_a$, $-\text{CN}$, and $-\text{NO}_2$, with the proviso that at least one E is an electron withdrawing group; and LG is a leaving group.

[0050] In at least one embodiment, the compound is a compound of Formula I:

- 10 -



wherein:

each X is independently C or N;

each E is independently hydrogen or an electron withdrawing group selected from the
 5 group consisting of $-\text{CF}_3$, $-\text{C}(\text{O})-\text{R}_a$, $-\text{SO}_2-\text{R}_a$, $-\text{CN}$, and $-\text{NO}_2$, with the
 proviso that at least one E is an electron withdrawing group;

LG is a leaving group;

R^1 and R^2 are each independently absent or selected from the group consisting of $-\text{R}_a$,
 $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; or R_1 and R_2 , together
 10 with the carbon atoms to which they are attached, form a cycloalkyl,
 $-\text{heterocycloalkyl}$, $-\text{aryl}$, or $-\text{heteroaryl}$;

R^3 is absent or selected from the group consisting of $-\text{R}_a$, $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; and

each R_a is independently selected from the group consisting of $-\text{H}$, $-\text{alkyl}$, $-\text{O-alkyl}$,
 15 $-\text{N-alkyl}$, $-\text{alkenyl}$, $-\text{O-alkenyl}$, $-\text{N-alkenyl}$, $-\text{alkynyl}$, $-\text{O-alkynyl}$, $-\text{N-alkynyl}$, $-\text{aryl}$, $-\text{O-aryl}$, $-\text{N-aryl}$, $-\text{heteroaryl}$, $-\text{O-heteroaryl}$, $-\text{N-heteroaryl}$,
 $-\text{cycloalkyl}$, $-\text{O-cycloalkyl}$, $-\text{N-cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{O-heterocycloalkyl}$, and $-\text{N-heterocycloalkyl}$.

[0051] As used herein, the term “alkyl” refers to a straight or branched,
 20 saturated aliphatic radical containing one to about twenty (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9,
 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 1-
 10, 1-11, 1-12, 1-13, 1-14, 1-15, 1-16, 1-17, 1-18, 1-19, 1-20, 2-3, 2-4, 2-5, 2-6,
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10 **[0052]** As used herein, the term “alkenyl” refers to a straight or branched aliphatic unsaturated hydrocarbon of formula C_nH_{2n} having from two to about twenty (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 2-11, 2-12, 2-13, 2-14, 2-15, 2-16, 2-17, 2-18, 2-19, 2-20, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-11, 3-12, 3-13, 3-14, 3-15, 3-16, 3-17, 3-18, 3-19, 3-20, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, 4-13, 4-14, 4-15, 4-16, 4-17, 4-18, 4-19, 4-20, 5-6, 5-7, 5-8, 5-9, 5-10, 5-11, 5-12, 5-13, 5-14, 5-15, 5-16, 5-17, 5-18, 5-19, 5-20, 6-7, 6-8, 6-9, 6-10, 6-11, 6-12, 6-13, 6-14, 6-15, 6-16, 6-17, 6-18, 6-19, 6-20, 7-8, 7-9, 7-10, 7-11, 7-12, 7-13, 7-14, 7-15, 7-16, 7-17, 7-18, 7-19, 7-20, 8-9, 8-10, 8-11, 8-12, 8-13, 8-14, 8-15, 8-16, 8-17, 8-18, 8-19, 8-20, 9-10, 9-11, 9-12, 9-13, 9-14, 9-15, 9-16, 9-17, 9-18, 9-19, 9-20, 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 11-12, 11-13, 11-14, 11-15, 11-16, 11-17, 11-18, 11-19, 11-20, 12-13, 12-14, 12-15, 12-16, 12-17, 12-18, 12-19, 12-20, 13-14, 13-15, 13-16, 13-17, 13-18, 13-19, 13-20, 14-15, 14-16, 14-17, 14-18, 14-19, 14-20, 15-16, 15-17, 15-18, 15-19, 15-20, 16-17, 16-18, 16-19, 16-20, 17-18, 17-19, 17-20, 18-19, 18-20, 19-20) carbon atoms in the chain and, unless otherwise indicated, may be optionally substituted. Exemplary alkenyls include, without limitation, ethylenyl, propylenyl, *n*-butylenyl, and *i*-butylenyl.

[0053] As used herein, the term “alkynyl” refers to a straight or branched aliphatic unsaturated hydrocarbon of formula C_nH_{2n-2} having from two to about twenty (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 2-11, 2-12, 2-13, 2-14, 2-15, 2-16, 2-17, 2-18, 2-19, 2-20, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-11, 3-12, 3-13, 3-14, 3-15, 3-16, 3-17, 3-18, 3-19, 3-20, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, 4-13, 4-14, 4-15,

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[0054] As used herein, the terms “perfluoroalkyl”, “perfluoroalkenyl”, and “perfluoroalkynyl” refer to an alkyl, alkenyl, or alkynyl group as defined above in which the hydrogen atoms on at least one of the carbon atoms have all been replaced with fluorine atoms.

[0055] As used herein, the term “cycloalkyl” refers to a non-aromatic saturated or unsaturated monocyclic or polycyclic ring system which may contain 3 to 24 (3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 3-11, 3-12, 3-13, 3-14, 3-15, 3-16, 3-17, 3-18, 3-19, 3-20, 3-21, 3-22, 3-23, 3-24, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 4-11, 4-12, 4-13, 4-14, 4-15, 4-16, 4-17, 4-18, 4-19, 4-20, 4-21, 4-22, 4-23, 4-24, 5-6, 5-7, 5-8, 5-9, 5-10, 5-11, 5-12, 5-13, 5-14, 5-15, 5-16, 5-17, 5-18, 5-19, 5-20, 5-21, 5-22, 5-23, 5-24, 6-7, 6-8, 6-9, 6-10, 6-11, 6-12, 6-13, 6-14, 6-15, 6-16, 6-17, 6-18, 6-19, 6-20, 6-21, 6-22, 6-23, 6-24, 7-8, 7-9, 7-10, 7-11, 7-12, 7-13, 7-14, 7-15, 7-16, 7-17, 7-18, 7-19, 7-20, 7-21, 7-22, 7-23, 7-24, 8-9, 8-10, 8-11, 8-12, 8-13, 8-14, 8-15, 8-16, 8-17, 8-18, 8-19, 8-20, 8-21, 8-22, 8-23, 8-24, 9-10, 9-11, 9-12, 9-13, 9-14, 9-15, 9-16, 9-17, 9-18, 9-19, 9-20, 9-21, 9-22, 9-23, 9-24, 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 10-21, 10-22, 10-23, 10-24, 11-12, 11-13, 11-14, 11-15, 11-16, 11-17, 11-18, 11-19, 11-20, 11-21, 11-22, 11-23, 11-24, 12-13, 12-14, 12-15, 12-16, 12-17, 12-18, 12-19, 12-20, 12-21, 12-22, 12-23, 12-24, 13-14, 13-15, 13-16, 13-17, 13-18, 13-19, 13-20, 13-21, 13-22, 13-23,

13-24, 14-15, 14-16, 14-17, 14-18, 14-19, 14-20, 14-21, 14-22, 14-23, 14-24, 15-16, 15-17, 15-18, 15-19, 15-20, 15-21, 15-22, 15-23, 15-24, 16-17, 16-18, 16-19, 16-20, 16-21, 16-22, 16-23, 16-24, 17-18, 17-19, 17-20, 17-21, 17-22, 17-23, 17-24, 18-19, 18-20, 18-21, 18-22, 18-23, 18-24, 19-20, 19-21, 19-22, 19-23, 19-24, 20-21, 20-22, 20-23, 20-24, 21-22, 22-23, 22-24, 23-24) carbon atoms, which may include at least one double bond and, unless otherwise indicated, the ring system may be optionally substituted. Exemplary cycloalkyl groups include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclohexenyl, *anti*-bicyclopropane, and *syn*-bicyclopropane.

10 **[0056]** As used herein, the term “heterocycloalkyl” refers to a cycloalkyl group as defined above having at least one O, S, and/or N interrupting the carbocyclic ring structure. Examples of heterocycloalkyls include, without limitation, piperidine, piperazine, morpholine, thiomorpholine, pyrrolidine, tetrahydrofuran, pyran, tetrahydropyran, and oxetane. Unless otherwise indicated, the heterocycloalkyl ring system may be optionally substituted.

15 **[0057]** As used herein, the term “aryl” refers to an aromatic monocyclic or polycyclic ring system from 6 to 24 (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 6-7, 6-8, 6-9, 6-10, 6-11, 6-12, 6-13, 6-14, 6-15, 6-16, 6-17, 6-18, 6-19, 6-20, 6-21, 6-22, 6-23, 6-24, 7-8, 7-9, 7-10, 7-11, 7-12, 7-13, 7-14, 7-15, 7-16, 7-17, 7-18, 7-19, 7-20, 7-21, 7-22, 7-23, 7-24, 8-9, 8-10, 8-11, 8-12, 8-13, 8-14, 8-15, 8-16, 8-17, 8-18, 8-19, 8-20, 8-21, 8-22, 8-23, 8-24, 9-10, 9-11, 9-12, 9-13, 9-14, 9-15, 9-16, 9-17, 9-18, 9-19, 9-20, 9-21, 9-22, 9-23, 9-24, 10-11, 10-12, 10-13, 10-14, 10-15, 10-16, 10-17, 10-18, 10-19, 10-20, 10-21, 10-22, 10-23, 10-24, 11-12, 11-13, 11-14, 11-15, 11-16, 11-17, 11-18, 11-19, 11-20, 11-21, 11-22, 11-23, 11-24, 12-13, 12-14, 12-15, 12-16, 12-17, 12-18, 12-19, 12-20, 12-21, 12-22, 12-23, 12-24, 13-14, 13-15, 13-16, 13-17, 13-18, 13-19, 13-20, 13-21, 13-22, 13-23, 13-24, 14-15, 14-16, 14-17, 14-18, 14-19, 14-20, 14-21, 14-22, 14-23, 14-24, 15-16, 15-17, 15-18, 15-19, 15-20, 15-21, 15-22, 15-23, 15-24, 16-17, 16-18, 16-19, 16-20, 16-21, 16-22, 16-23, 16-24, 17-18, 17-19, 17-20, 17-21, 17-22, 17-23, 17-24, 18-19, 18-20, 18-21, 18-22, 18-23, 18-24, 19-20, 19-21, 19-22, 19-23, 19-24, 20-21, 20-22, 20-23, 20-24, 21-22, 22-23, 22-24, 23-24) carbon atoms and, unless otherwise indicated, the ring system may be optionally substituted. Aryl groups of the present technology include, but are not limited to, groups such as

phenyl, naphthyl, azulenyl, phenanthrenyl, anthracenyl, fluorenyl, pyrenyl, triphenylenyl, chrysenyl, naphthacenyl, biphenyl, triphenyl, and tetraphenyl. In at least one embodiment, an aryl within the context of the present technology is a 6 or 10 membered ring. In at least one embodiment, each aryl is phenyl or naphthyl.

5 **[0058]** As used herein, the term “heteroaryl” refers to an aryl group as defined above having at least one O, S, and/or N interrupting the carbocyclic ring structure. Examples of heteroaryl groups include, without limitation, pyrrolyl, pyrazolyl, imidazolyl, triazolyl, furyl, thiophenyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, triazinyl, 10 thienopyrrolyl, furopyrrolyl, indolyl, azaindolyl, isoindolyl, indolyl, indolizyl, indazolyl, benzimidazolyl, imidazopyridinyl, benzotriazolyl, benzoxazolyl, benzoxadiazolyl, benzothiazolyl, pyrazolopyridinyl, triazolopyridinyl, thienopyridinyl, benzothiadiazolyl, benzofuyl, benzothiophenyl, quinolyl, isoquinolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, cinnolyl, quinazolyl, 15 quinolizilyl, phthalazinyl, benzotriazinyl, chromenyl, naphthyridinyl, acrydinyl, phenanzinyl, phenothiazinyl, phenoxazinyl, pteridinyl, and purinyl. Additional heteroaryls are described in COMPREHENSIVE HETEROCYCLIC CHEMISTRY: THE STRUCTURE, REACTIONS, SYNTHESIS AND USE OF HETEROCYCLIC COMPOUNDS (Katritzky et al. eds., 1984), which is hereby incorporated by reference in its entirety.

20 Unless otherwise indicated, the heteroaryl ring system may be optionally substituted.

[0059] The term “monocyclic” as used herein indicates a molecular structure having one ring.

[0060] The term “polycyclic” as used herein indicates a molecular structure having two or more rings, including, but not limited to, fused, bridged, spiro, or 25 covalently bound rings. In at least one embodiment, the polycyclic ring system is a bicyclic, tricyclic, or tetracyclic ring system. In at least one embodiment, the polycyclic ring system is fused. In at least one embodiment, the polycyclic ring system is a bicyclic ring system such as naphthyl or biphenyl.

[0061] As used herein, the term “optionally substituted” indicates that a group 30 may have a substituent at each substitutable atom of the group (including more than one substituent on a single atom), provided that the designated atom’s normal valency is not exceeded and the identity of each substituent is independent of the others. Up to three H atoms in each residue are replaced with alkyl, halogen, haloalkyl, hydroxy,

alkoxy (especially C₁–C₃), carboxy, ester, amide, carbonate, carbamate, urea, cyano, carbonyl, nitro, amino, alkylamino, dialkylamino, mercapto, alkylthio, sulfoxide, sulfone, acylamino, amidino, phenyl, benzyl, heteroaryl, phenoxy, benzyloxy, or heteroaryloxy. “Unsubstituted” atoms bear all of the hydrogen atoms dictated by their
 5 valency. When a substituent is keto (*i.e.*, =O), then two hydrogens on the atom are replaced. Combinations of substituents and/or variables are permissible only if such combinations result in stable compounds; by “stable compound” is meant a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into an efficacious agent.

10 **[0062]** As used herein, the term “halogen” includes fluorine, bromine, chlorine, and iodine while the term “halide” includes fluoride, bromide, chloride, and iodide anion.

[0063] In at least one embodiment of the compounds of the present technology, each X is C. In other embodiments, one or more X moieties are N. One, two, three,
 15 or four of the X moieties may be N. For example, when one X moiety is N, a pyridine ring is provided in the compound, and when two X moieties are N, a pyridazine, pyrimidine, or pyrazine ring is provided in the compound. In a particularly preferred embodiment of the present technology, each X is C, providing a phenyl ring.

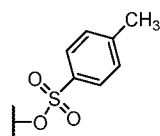
[0064] In at least one embodiment of the compounds of the present technology,
 20 each E is independently an electron withdrawing group. Suitable electron withdrawing groups include –CF₃, –C(O)–R_a, –SO₂–R_a, –CN, and –NO₂, where each R_a is independently selected from the group consisting of –H, –alkyl, –O–alkyl, –N–alkyl, –alkenyl, –O–alkenyl, –N–alkenyl, –alkynyl, –O–alkynyl, –N–alkynyl, –aryl, –O–aryl, –N–aryl, –heteroaryl, –O–heteroaryl, –N–heteroaryl, –cycloalkyl, –O–
 25 cycloalkyl, –N–cycloalkyl, –heterocycloalkyl, –O–heterocycloalkyl, and –N–heterocycloalkyl. In a particularly preferred embodiment, each E is –NO₂.

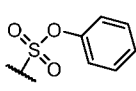
[0065] LG in the compounds of the present technology is a leaving group. Suitable leaving groups are substituents that are present on the compound that can be displaced (preferably for nucleophilic aromatic substitution). Suitable leaving groups
 30 are apparent to a skilled artisan. In at least one embodiment, the leaving group is selected from the group consisting of halogen (*e.g.*, –I, –Br, –Cl, –F), –OR_b, –OC(O)R_b, –OS(O)₂R_b, –S(O)₂–O–R_b, –N₂⁺, –N⁺(R_b)₃, –S⁺(R_b)₂, and –P⁺(R_b)₃; where each R_b is independently selected from the group consisting of hydrogen, –alkyl, –O–

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alkyl, -N-alkyl, -alkenyl, -O-alkenyl, -N-alkenyl, -alkynyl, -O-alkynyl, -N-alkynyl, -perfluoroalkyl, -perfluoroalkenyl, -perfluoroalkynyl, -aryl, -O-aryl, -N-aryl, -heteroaryl, -O-heteroaryl, -N-heteroaryl, -cycloalkyl, -O-cycloalkyl, -N-cycloalkyl, -heterocycloalkyl, -O-heterocycloalkyl, and -N-heterocycloalkyl. In at

5 least one embodiment, the leaving group is , , , or

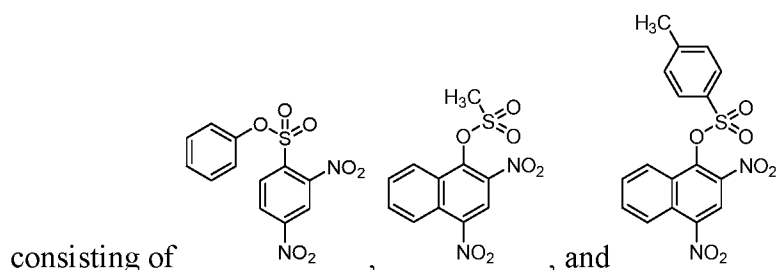


. In a preferred embodiment, the leaving group is .

[0066] In certain embodiments, R^1 and R^2 in the compound of Formula I are each independently absent or selected from the group consisting of - R_a , -cycloalkyl, -heterocycloalkyl, -aryl, and -heteroaryl. In other embodiments, R_1 and R_2 , together with the carbon atoms to which they are attached, form a cycloalkyl, heterocycloalkyl, aryl, or heteroaryl. In at least one embodiment, R_1 and R_2 , together with the carbon atoms to which they are attached, form a naphthalene, benzimidazole, quinoline, or indole ring. In a preferred embodiment, R_1 and R_2 , together with the carbon atoms to which they are attached, form a naphthalene ring.

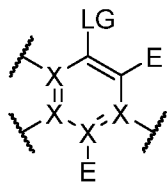
15 [0067] In at least one embodiment, R^1 , R^2 , and R^3 of the compound of Formula I are each hydrogen.

[0068] In at least one embodiment, the compound is selected from the group



[0069] Another aspect of the present technology relates to an analytical method. This method involves contacting a sample potentially containing D-cysteine, L-cysteine, or a mixture thereof with a probe under conditions effective to result in double *ipso*-substitution of any cysteine present in the sample and determining, based on any double *ipso*-substituted cysteine that forms, the absolute configuration of D/L-cysteine in the sample, and/or the enantiomeric composition of cysteine in the sample, and/or the concentration of total cysteine in the sample.

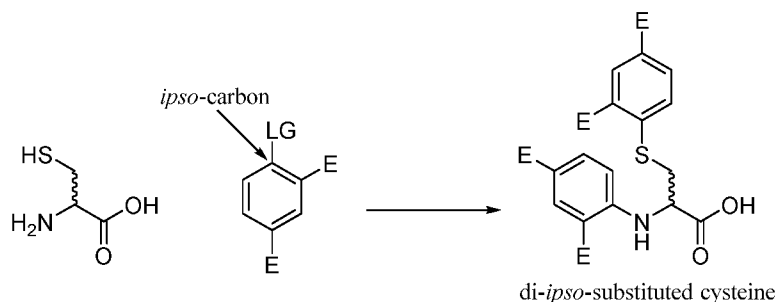
[0070] Suitable probes for use in the methods of the present technology include the compounds comprising a chromophore of the formula



(including compounds of Formula I and all embodiments thereof)

described herein.

- 5 [0071] As used herein, “double *ipso*-substitution,” or “double *ipso*-substituted” refers to the disubstitution reaction between free-form cysteine and probe of the present technology. By way of example, the following scheme presents a general overview of a double *ipso*-substitution reaction.



- 10 [0072] As used herein, the term “absolute configuration” of D/L-cysteine refers to the identification of the major cysteine enantiomer present in a sample. The absolute configuration of D/L-cysteine can be assigned from the chiroptical signal of the double *ipso*-substituted cysteine complexes that form. This assignment can be based on the sense of chirality induction with a reference or by analogy. The absolute
- 15 configuration of the major cysteine enantiomer is indicated by the sign of the produced Cotton effect. The chiroptical signal of the complexes can be measured using standard techniques, which will be apparent to the skilled artisan. Such techniques include circular dichroism spectroscopy (*e.g.*, STEREOCHEMISTRY OF ORGANIC COMPOUNDS 1003–07 (E. L. Eliel & S.H. Wilen eds., 1994);
- 20 DYNAMIC STEREOCHEMISTRY OF CHIRAL COMPOUNDS 140–43 (Christian Wolf ed., 2008), each of which is hereby incorporated by reference in its entirety), optical rotatory dispersion (*e.g.*, STEREOCHEMISTRY OF ORGANIC COMPOUNDS 999–1003 (E. L. Eliel & S. H. Wilen eds., 1994), which is hereby incorporated by reference in its entirety), and polarimetry (*e.g.*,
- 25 STEREOCHEMISTRY OF ORGANIC COMPOUNDS 217–21, 1071–80 (E. L. Eliel

& S. H. Wilen eds., 1994); DYNAMIC STEREOCHEMISTRY OF CHIRAL COMPOUNDS 140– 43 (Christian Wolf ed., 2008), each of which is hereby incorporated by reference in its entirety). As will be apparent to the skilled artisan, absolute configuration can be determined by reference to a standard. By way of

5 example, a standard could be the optical spectra (or information regarding the sign of the Cotton effect at relevant wavelengths produced thereby) obtained from standard samples generated by mixing enantiomerically pure samples of each enantiomer of cysteine with a probe (*i.e.*, the particular probe to be used with the test sample or a probe that would produce substantially the same results). The chiroptical signal of the

10 test sample can be measured by generating an optical spectrum of the double *ipso*-substituted cysteine in the test sample under analogous conditions. The absolute configuration of the major cysteine enantiomer originally present in the test sample can then be determined by comparing the optical spectrum of the test sample to that of the standard sample(s). As will be apparent to the skilled artisan, different probes,

15 reaction conditions (solvent, etc.), and detection methods (circular dichroism, optical rotatory dispersion, or polarimetry) may produce different Cotton effects with regard to the amplitude, shape, sign, and relevant wavelengths. Those skilled in the art will know how to adjust these factors so that the test conditions sufficiently match the conditions used to generate the standard.

20 **[0073]** As used herein, determining the “enantiomeric composition” of a sample refers to determining: (1) the enantiomeric excess of D-cysteine, (2) the enantiomeric excess of L-cysteine, (3) the enantiomeric excess of both D-cysteine and L-cysteine, (4) the enantiomeric ratio of D:L cysteine, (5) the enantiomeric ratio of L:D cysteine, or (5) any combination thereof. The term “enantiomeric ratio” (er) is

25 the ratio of the percentage of one cysteine enantiomer in a mixture to that of the other enantiomer. The term “enantiomeric excess” (ee) is the difference between the percentage of one cysteine enantiomer and the percentage of the other cysteine enantiomer. For example, a sample which contains 75% L-cysteine and 25% D-cysteine will have an enantiomeric excess of 50% of L- cysteine and an enantiomeric

30 ratio (D:L) of 25:75. The enantiomeric excess/ratio can be determined by correlating the chiroptical signal of the double *ipso*-substituted cysteine complexes that form to the enantiomeric excess/ratio of the analyte(s). The enantiomeric composition is based on the induced amplitude of chiroptical signal. The chiroptical signal can be

measured using standard techniques, which will be apparent to the skilled artisan. Such techniques include circular dichroism spectroscopy (*e.g.*, STEREOCHEMISTRY OF ORGANIC COMPOUNDS 1003–07 (E. L. Eliel & S. H. Wilen eds., 1994); DYNAMIC STEREOCHEMISTRY OF CHIRAL COMPOUNDS 140–43 (Christian Wolf ed., 2008), each of which is hereby incorporated by reference in its entirety), optical rotatory dispersion (*e.g.*, STEREOCHEMISTRY OF ORGANIC COMPOUNDS 999–1003 (E. L. Eliel & S. H. Wilen eds., 1994), which is hereby incorporated by reference in its entirety), and polarimetry (*e.g.*, STEREOCHEMISTRY OF ORGANIC COMPOUNDS 217–21, 1071–80 (E. L. Eliel & S. H. Wilen eds., 1994); DYNAMIC STEREOCHEMISTRY OF CHIRAL COMPOUNDS 140–43 (Christian Wolf ed., 2008), each of which is hereby incorporated by reference in its entirety). As will be apparent to the skilled artisan, enantiomeric composition can be determined by reference to a standard. By way of example, a standard could be the optical spectra (or information regarding the induced amplitude of the signal at relevant wavelengths produced thereby) obtained from standard samples generated by mixing enantiomerically pure samples of each enantiomer of cysteine with a probe (*i.e.*, the particular probe to be used with the test sample or a probe that would produce substantially the same results). The chiroptical signal of the test sample can be measured by generating an optical spectrum of the double *ipso*-substituted cysteine in the test sample under analogous conditions. The enantiomeric composition of the cysteine originally present in the sample can then be determined by comparing the optical spectrum of the test sample to that of the standard sample(s) and performing the appropriate mathematical calculation(s) to determine the ee/er of the enantiomer(s) of interest. As noted above, different probes, reaction conditions (solvent, etc.), and detection methods (circular dichroism, optical rotatory dispersion, or polarimetry) may produce different Cotton effects with regard to the amplitude, shape, sign, and relevant wavelengths. Those skilled in the art will know how to adjust these factors so that the test conditions sufficiently match the conditions used to generate the standard.

30 **[0074]** The total concentration of cysteine (*i.e.*, D- plus L-) can be determined by correlating a non-chiroptical spectroscopic signal of the double *ipso*-substituted cysteine complexes that form to the concentration of total cysteine. The non-chiroptical spectroscopic signal can be measured using standard techniques, which

will be apparent to the skilled artisan. Such techniques include, but are not limited to, UV (including UV/Vis) spectroscopy (PRINCIPLES OF INSTRUMENTAL ANALYSIS 342–47 (Douglas A. Skoog et al. eds., 5th ed. 1998), which is hereby incorporated by reference in its entirety), fluorescence spectroscopy, and other spectroscopic techniques. By way of example, a standard could be the spectroscopic spectra (*e.g.*, UV, fluorescence) (or relevant information about the spectra) obtained from standard samples generated by mixing serial titrations of cysteine with a probe (*i.e.*, the particular probe to be used with the test sample or a probe that would produce substantially the same results). The spectroscopic signal (*e.g.*, UV, fluorescence) of the double *ipso*-substituted cysteine can be measured by generating a spectrum (*e.g.*, UV, fluorescence) of the test sample under analogous conditions. The total concentration of the cysteine originally present in the sample can then be determined by comparing the spectrum of the test sample to the titration curve of the standard samples (or relevant values obtained from the titration curve). As will be apparent to the skilled artisan, if the enantiomeric ratio of cysteine is also determined, the concentration of individual enantiomers originally present in the test sample can be determined by comparing the enantiomeric excess to the total cysteine concentration.

[0075] In at least one embodiment of the present technology, the absolute configuration of D/L-cysteine in the sample is determined. In another embodiment, the enantiomeric composition of cysteine in the sample is determined. In another embodiment, the concentration of total cysteine in the sample is determined.

[0076] In at least one preferred embodiment of the present technology, more than one of the absolute configuration, enantiomeric composition, and total cysteine concentration are determined. For example, the analytical method of the present technology may be used to determine the absolute configuration of D/L-cysteine in the sample and the concentration of total cysteine in the sample. Alternatively, the analytical method of the present technology may be used to determine the enantiomeric composition of cysteine in the sample and the concentration of total cysteine in the sample. In a preferred embodiment, the analytical method of the present technology is used to determine the absolute configuration of D/L-cysteine in the sample, the enantiomeric composition of cysteine in the sample, and the concentration of total cysteine in the sample. In a particularly preferred embodiment,

the absolute configuration of D/L-cysteine in the sample, the enantiomeric composition of cysteine in the sample, and the concentration of total cysteine in the sample are all determined concomitantly (*i.e.*, by obtaining a chiroptical signal (*e.g.*, CD) and a non-chiroptical spectroscopic signal (*e.g.*, UV) simultaneously or substantially simultaneously).

5 [0077] The methods of the present technology can be used to determine the absolute configuration, enantiomeric composition, and/or total cysteine concentration in a sample even when the cysteine is present at low concentrations. In at least one embodiment, the analytical method of the present technology is carried out on a sample in which the cysteine is present in micromolar concentrations. The analytical methods of the present technology may also be carried out on samples in which the cysteine concentration is above or below the micromolar range. In at least one embodiment, the sample contains cysteine in the nanomolar range and the absolute configuration of D/L cysteine in the sample, the total concentration of cysteine in the sample, or both are determined.

15 [0078] The analytical methods of the present technology can be used to determine the characteristics (absolute configuration, enantiomeric composition, and/or total cysteine concentration) of free-form cysteine in a sample. The analytical methods of the present technology can selectively detect free-form cysteine even in samples containing other biological agents, such as proteins (including proteins containing cysteine), other free-form amino acids, and other biological thiols such as L-homocysteine and L-glutathione.

20 [0079] Suitable samples include any aqueous or non-aqueous solution that potentially contains D-cysteine, L-cysteine, or a mixture thereof. In a preferred embodiment, the sample is an aqueous solution. The solution may be biological or non-biological. As will be apparent to the skilled artisan, an otherwise solid sample can be suspended in, mixed with, etc., a liquid vehicle to produce a suitable aqueous or non-aqueous solution and then tested.

25 [0080] In at least one embodiment, the sample is a biological sample from an animal. Suitable biological samples include, without limitation, blood, plasma, brain extracellular fluid, cerebrospinal fluid, tissue, cells, cell extracts, cell lysates, serum, semen, amniotic fluid, sputum, urine, feces, bodily fluids, bodily secretions, bodily excretions, circulating tumor cells, tumor, tumor biopsy, and exosomes. In at least

one preferred embodiment, the sample is brain extracellular fluid or cerebrospinal fluid.

[0081] Suitable animals include, for example, vertebrates, *e.g.*, mammals, fish, reptiles, birds, and amphibians. Suitable mammals include, for example, primates, felines, canines, equines, camelids, bovines, caprines, ovines, swine, rabbits, and rodents. For example, the methods of the present technology can be used to analyze cysteine in a sample from a human subject, a laboratory animal (*e.g.*, monkeys, chimpanzees, rabbits, rats, mice, guinea pigs, hamsters, zebrafish, frogs), or a veterinary animal, such as livestock (*e.g.*, cattle, sheep, pigs, goats, horses, camels, llamas, alpacas, rodents) and pets (*e.g.*, cats, dogs). In a preferred embodiment, the animal is a human subject.

[0082] As will be apparent to the skilled artisan, the present methods are especially suitable for evaluating cysteine wherever cysteine plays an important biological function in its free form. For example, in at least one preferred embodiment, the sample is a biological sample of a patient (human or other animal) having or suspected of having or at risk of having a disorder mediated by free form cysteine.

[0083] By way of example, free form cysteine plays an important biological function in the brain and has been implicated in neurodegenerative diseases. In at least one embodiment, the sample is a biological sample of a patient having or suspected of having or at risk of having a neurodegenerative disease. Without being bound by any particular theory, it is envisioned that the present technology has utility in identifying and/or detecting and/or monitoring the pathology of a neurodegenerative disease in a human subject by detecting the absolute configuration of D/L-cysteine, and/or the enantiomeric composition of cysteine, and/or the concentration of total cysteine in a neurological sample, such as brain extracellular fluid or cerebrospinal fluid. Examples of neurodegenerative diseases include, but are not limited to, amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD), Parkinson's disease, Huntington's disease, mild cognitive impairment (MCI), Alzheimer's disease, and diseases associated with TPD-43 proteinopathy.

[0084] Free form cysteine has also been implicated in metabolic syndrome (including, without limitation, central adiposity, hyperglycemia, hypertension, dyslipidemia, and insulin resistance), diabetes (*e.g.*, Type 2 diabetes), obstructive sleep

- apnea, irritable bowel disease/inflammatory bowel disease, and diseases associated with skeletal wasting or muscle fatigue (*e.g.*, HIV/SIV infection, cancer, major injuries, sepsis, Crohn's disease, ulcerative colitis, and chronic fatigue syndrome). (*See* Mohorco et al., *BioMed Res. Int.* Article ID 418681 (2015); Cintra et al., *Chest* 139(2):246–52 (2011);
- 5 Dröge et al., *FASEB J.* 11(13):1077–89 (1997); Hack et al., *FASEB J.* 11(1):84–92 (1997); Jain, *European J. Clin. Nutr.* 68:1148–53 (2014); Sido et al., *Gut* 42(4): 485–92 (1998), each of which is hereby incorporated by reference in its entirety.) In at least one embodiment, the sample is a biological sample of a patient having or suspected of having or at risk of having any of these disorders. Without being bound by any particular
- 10 theory, it is envisioned that the present technology has utility in identifying and/or detecting and/or monitoring the pathology of any of these disorders in a subject by detecting the absolute configuration of D/L-cysteine, and/or the enantiomeric composition of cysteine, and/or the concentration of total cysteine in a relevant biological sample (especially blood, plasma, or serum).
- 15 **[0085]** The analytical method may further comprise the steps of providing the sample and providing the probe. Suitable ways of obtaining test samples will be readily apparent to the skilled artisan. As will also be apparent to the skilled artisan, probes may be made using (or adapting as needed), the synthesis strategies described in the Examples.
- 20 **[0086]** The present technology may be further illustrated by reference to the following examples.

EXAMPLES

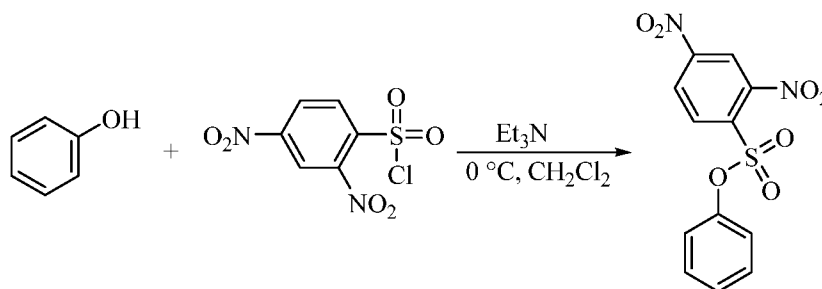
- [0087]** The following examples are provided to illustrate embodiments of the present technology, but they are by no means intended to limit its scope.
- 25 **Example 1—General Materials and Methods**
- [0088]** *Synthesis.* Probes **1**, **2**, and **3** described herein were synthesized using literature procedures. All reagents and solvents were commercially available and used without further purification. Reactions were carried out under inert conditions and the products were purified by flash chromatography on silica gel. The structures
- 30 of the isolated compounds were identified by spectroscopic analyses and the purity was confirmed by elemental analysis. Further details are described in Example 2.

[0089] **Crystallographic Analysis.** Single crystals of **1**, **2** and **3** were obtained by slow evaporation of solutions in acetonitrile or chloroform. Single crystal X-ray analysis was performed at 296 K using a Siemens platform diffractometer with graphite monochromated Mo-K α radiation ($\lambda = 0.71073$ Å). Data were integrated and corrected using the Apex 2 program. The structures were solved by direct methods and refined with full-matrix least-square analysis using SHELX-97-2 software. Non-hydrogen atoms were refined with anisotropic displacement parameter. More details and crystallographic data can be found in Example 3.

[0090] **Spectroscopic Analysis.** ^1H -NMR and ^{13}C -NMR spectra, including DEPT and 2D NMR measurements, were obtained at 400 MHz and 100 MHz, respectively, using deuterated acetone, chloroform, or acetonitrile/water mixtures as solvents. Chemical shifts were reported in ppm relative to TMS or the solvent peak. CD spectra were collected with a standard sensitivity of 100 mdeg, a data pitch of 0.5 nm, a bandwidth of 1 nm, in a continuous scanning mode with a scanning speed of 500 nm/min and a response of 1 s, using a quartz cuvette (1 cm path length). The data were baseline corrected and smoothed using a binomial equation. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm, and a scan rate of 400 nm/s. Full range NMR spectra, CD spectra, and UV spectra together with detailed information on sample preparation are provided in the subsequent Examples.

Example 2—Synthesis and Characterization of Cysteine Probes and *N,S*-Bis(2,4-Dinitrophenyl)Cysteine

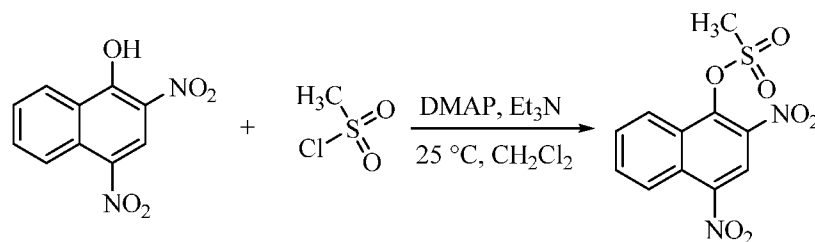
Synthesis of phenyl 2,4-dinitrobenzenesulfonate (**1**)



[0091] A mixture of phenol (282.3 mg, 3.0 mmol) and triethylamine (460 μL , 3.3 mmol) was stirred in anhydrous CH_2Cl_2 (2 mL) for 1 hour at 0 °C. Then, 2,4-dinitrosulfonyl chloride (1.32 g, 4.95 mmol) in CH_2Cl_2 (3 mL) was added dropwise to

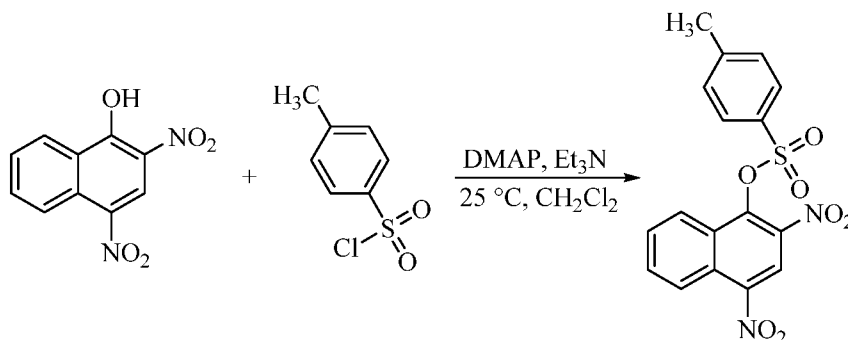
the reaction mixture. After stirring for 18 hours, the reaction was quenched with water and extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄ and concentrated in vacuo. Purification by flash chromatography on silica gel (10% EtOAc in hexanes) afforded 833.5 mg (2.57 mmol, 86% yield) of a light yellow solid. ¹H NMR (400 MHz, CDCl₃): δ = 8.65 (d, *J* = 2.2 Hz, 1H), 8.48 (dd, *J* = 8.6 Hz, 2.1 Hz, 1H), 8.17 (d, *J* = 8.6 Hz, 1H), 7.44 – 7.30 (m, 3H), 7.20 (d, *J* = 7.9 Hz, 2H). ¹³C NMR (100 MHz, CDCl₃): δ = 120.4, 122.1, 126.41, 128.4, 130.4, 133.8, 134.0, 149.0, 151.0. Anal. Calcd. for C₁₂H₈N₂O₇S: C, 44.45; H, 2.49; N, 8.64. Found: C, 44.59; H, 2.58; N, 8.51. See Figures 5A–B.

10 *Synthesis of 2,4-dinitronaphthalen-1-yl methanesulfonate (2)*



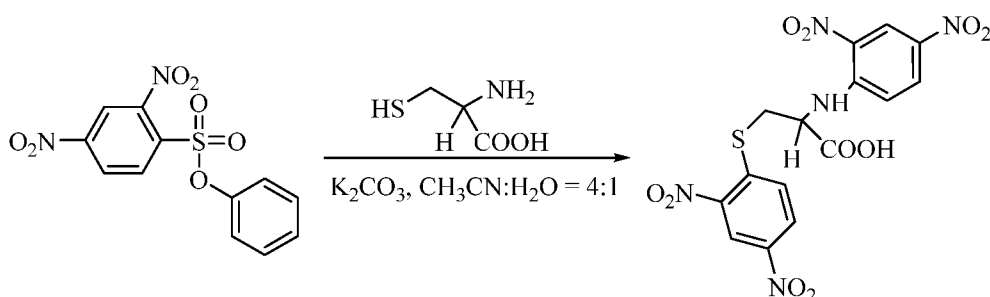
[0092] Methanesulfonyl chloride (46.0 μL, 0.593 mmol) was added dropwise to a solution of a 2,4-dinitronaphth-1-ol (123.9 mg, 0.539 mmol), triethylamine (82.7 μL, 0.593 mmol), and DMAP (6.6 mg, 0.054 mmol) in CH₂Cl₂ (2 mL) at room temperature. After the reaction was complete, the mixture was washed with water and extracted with CH₂Cl₂. The combined organic layers were dried over MgSO₄ and concentrated in vacuo. Purification by flash column chromatography on silica gel (20% EtOAc in hexanes) afforded 84.0 mg (0.27 mmol, 54%) of a yellow solid. ¹H NMR (400 MHz, CDCl₃): δ = 8.85 (s, 1H), 8.67–8.58 (m, 2H), 7.99 (ddd, *J* = 8.7, 7.0, 1.3 Hz, 1H), 7.91 (ddd, *J* = 8.1, 7.0, 1.1 Hz, 1H), 3.62 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ = 40.7, 119.4, 123.6, 126.1, 128.0, 130.0, 130.3, 133.4, 142.3. Anal. Calcd. for C₁₁H₈N₂O₇S: C, 42.31; H, 2.58; N, 8.97. Found: C, 42.41; H, 2.70; N, 8.85. See Figures 6A–B.

Synthesis of 2,4-dinitronaphthalen-1-yl 4-toluenesulfonate (3)



[0093] *p*-Toluenesulfonic acid (152.9 mg, 0.802 mmol) was added dropwise to a solution of 2,4-dinitronaphth-1-ol (170.8 mg, 0.729 mmol), triethylamine (112 μ L, 0.802 mmol), and DMAP (8.9 mg, 0.073 mmol) in CH_2Cl_2 (2 mL) at room temperature. After the reaction was complete, the mixture was washed with water and extracted with CH_2Cl_2 . The combined organic layers were dried over MgSO_4 and concentrated in vacuo. Purification by flash column chromatography on silica gel (20% EtOAc in hexanes followed by 10% MeOH in CH_2Cl_2) afforded 137.2 mg (0.35 mmol, 55%) of a yellow solid. ^1H NMR (400 MHz, CDCl_3): δ = 8.76 (s, 1H), 8.62 (dd, J = 8.9, 0.9 Hz, 1H), 8.35 (dd, J = 8.7, 1.0 Hz, 1H), 7.93 (ddd, J = 8.4, 7.0, 1.3 Hz, 1H), 7.90-7.87 (m, 2H), 7.76 (ddd, J = 8.3, 7.0, 1.1 Hz, 1H), 7.49-7.39 (m, 2H), 2.52 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ = 21.9, 119.4, 123.5, 125.8, 127.8, 128.8, 129.7, 129.8, 130.4, 131.5, 133.0, 142.4, 144.4, 147.2. Anal. Calcd. for $\text{C}_{17}\text{H}_{12}\text{N}_2\text{O}_7\text{S}$: C, 52.58; H, 3.11; N, 7.21. Found: C, 52.72; H, 3.41; N, 6.82. See Figures 7A–B.

Synthesis of *N,S*-bis(2,4-dinitrophenyl)cysteine (4)



20

A solution of phenyl 2,4-dinitrobenzenesulfonate (**1**) (100.0 mg, 0.309 mmol) in 2 mL of CH_3CN was added to a mixture of cysteine (18.7 mg, 0.154 mmol) and K_2CO_3 (85.1 mg, 0.616 mmol) in 0.5 mL of deionized water at room temperature. After the

reaction was complete, the mixture was concentrated in vacuo. Column purification on silica gel using 100% ethyl acetate followed by 5% formic acid in ethyl acetate as mobile phase afforded 49.9 mg (110 μ Mol, 72%) of an amorphous yellow solid. ^1H NMR (400 MHz, $(\text{CD}_3)_2\text{CO}$) δ = 9.04 (d, J = 7.7 Hz, 1H), 8.90 (d, J = 2.7 Hz, 1H), 8.83 (d, J = 2.5 Hz, 1H), 8.34 (dd, J = 9.0, 2.5 Hz, 1H), 8.29 (dd, J = 9.5, 2.7 Hz, 1H), 7.38 (d, J = 9.5 Hz, 1H), 5.31 (m, 1H), 4.17 (dd, J = 14.0, 4.4 Hz, 1H), 4.00 (dd, J = 14.0, 6.3 Hz, 1H). ^{13}C NMR (100 MHz, $(\text{CD}_3)_2\text{CO}$) δ = 34.3, 54.5, 115.3, 120.9, 123.3, 127.0, 129.2, 130.0, 136.9, 143.3, 144.6, 146.4, 146.8, 146.9, 169.5. See Figures 8A–B. (See also Figures 9–13 for COSY, HSQC, HMBC, DEPT, and NOESY spectra, respectively.)

Example 3—Crystallographic Analysis

[0094] *Phenyl 2,4-dinitrobenzenesulfonate (1)*. A single crystal was obtained by slow evaporation of a solution of phenyl 2,4-dinitrobenzenesulfonate in CH_3CN . Single crystal X-ray analysis was performed at 296 K using a Siemens platform diffractometer with graphite monochromated Mo- $\text{K}\alpha$ radiation (λ = 0.71073 Å). Data were integrated and corrected using the Apex 2 program. The structures were solved by direct methods and refined with full-matrix least-square analysis using SHELX-97-2 software. Non-hydrogen atoms were refined with anisotropic displacement parameter. Crystal data: $\text{C}_{12}\text{H}_8\text{N}_2\text{O}_7\text{S}$, M = 324.26, colorless needle, 0.8 x 0.6 x 0.3 mm³, orthorhombic, space group $P2_12_1$, a = 6.2524(10), b = 10.8770(18), c = 19.422(3) Å, V = 1320.8(4) Å³, Z = 4. Absolute structure parameter is not given since crystal was achiral that crystallized in a chiral space group. The crystal structure of probe **1** is shown in Figure 14.

[0095] *2,4-Dinitronaphthalen-1-yl methanesulfonate (2)*. A single crystal was obtained by slow evaporation of a solution of 2,4-dinitronaphthalen-1-yl methanesulfonate in CHCl_3 . Single crystal X-ray analysis was performed at 100 K using a Siemens platform diffractometer with graphite monochromated Mo- $\text{K}\alpha$ radiation (λ = 0.71073 Å). Data were integrated and corrected using the Apex 2 program. The structures were solved by direct methods and refined with full-matrix least-square analysis using SHELX-97-2 software. Non-hydrogen atoms were refined with anisotropic displacement parameter. Crystal data: $\text{C}_{11}\text{H}_8\text{N}_2\text{O}_7\text{S}$, M = 312.25, colorless needle, 0.4 x 0.7 x 0.9 mm³, monoclinic, space group $P2_1/c$, a = 9.3945(5), b

- 28 -

= 16.2435(8), $c = 8.0481(4)$ Å, $V = 1226.89(11)$ Å³, $Z = 4$. The crystal structure of probe **2** is shown in Figure 15.

[0096] **2,4-Dinitronaphthalen-1-yl 4-toluenesulfonate (3).** A single crystal was obtained by slow evaporation of a solution of 2,4-dinitronaphthalen-1-yl 4-toluenesulfonate in CHCl₃. Single crystal X-ray analysis was performed at 173 K using a Siemens platform diffractometer with graphite monochromated Mo-K α radiation ($\lambda = 0.71073$ Å). Data were integrated and corrected using the Apex 2 program. The structures were solved by direct methods and refined with full-matrix least-square analysis using SHELX-97-2 software. Non-hydrogen atoms were refined with anisotropic displacement parameter. Crystal data: C₁₇H₁₂N₂O₇S, $M = 388.35$, colorless needle, $0.3 \times 0.5 \times 1.0$ mm³, monoclinic, space group P2₁/c, $a = 12.8942(15)$, $b = 7.7595(9)$, $c = 16.4720(19)$ Å, $V = 1647.7(3)$ Å³, $Z = 4$. The crystal structure of probe **3** is shown in Figure 16.

Example 4—Probe Selection and Sensing Development

15 *Experimental Procedures*

[0097] **Generally.** Initially, reactions were performed with 1.25 mM cysteine concentrations as described below to identify a probe with superior chiroptical properties. The CD spectra of the diluted solutions (110 μ M) were collected with a standard sensitivity of 100 mdeg, a data pitch of 0.5 nm, a bandwidth of 1 nm, in a continuous scanning mode with a scanning speed of 500 nm/min and a response of 1 s, using a quartz cuvette (1 cm path length). The data were baseline corrected and smoothed using a binomial equation. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm, and a scan rate of 400 nm/s.

[0098] **Cysteine sensing using phenyl 2,4-dinitrobenzenesulfonate (1).** A solution of phenyl 2,4-dinitrobenzenesulfonate (**1**) (2.50 mM, 2.43 mg), cysteine (1.25 mM, 0.45 mg), and K₂CO₃ (5 mM, 2.07 mg) in 3 mL of CH₃CN:water (4:1) was stirred for 1 hour. To 200 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to CD analysis (110 μ M) (Figure 1B (left) and Figure 17). Control experiments with cysteine in the absence of the probe did not show any CD signal at the wavelengths of interest.

[0099] **Cysteine sensing using 2,4-dinitronaphthalen-1-yl methanesulfonate (2).** A solution of 2,4-dinitronaphthalen-1-yl methanesulfonate (**2**) (2.50 mM, 2.34

mg), cysteine (1.25 mM, 0.45 mg), and K_2CO_3 (5 mM, 2.07 mg) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. To 200 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to CD analysis (110 μ M) (Figure 18). Control experiments with cysteine in the absence of the probe did not show any CD signal at the wavelengths of interest.

[0100] Cysteine sensing using 2,4-dinitronaphthalen-1-yl 4-toluenesulfonate

(3). A solution of 2,4-dinitronaphthalen-1-yl-4-toluenesulfonate **(3)** (2.50 mM, 2.91 mg), cysteine (1.25 mM, 0.45 mg), and K_2CO_3 (5 mM, 2.07 mg) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. To 200 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to CD analysis (110 μ M) (Figure 1B (right) and Figure 19). Control experiments with cysteine in the absence of the probe did not show any CD signal at the wavelengths of interest.

[0101] A comparison of the CD spectra obtained with L-cysteine and probes **1–3** is shown in Figure 20.

[0102] Solvent and base optimization. A solution of probe **1** (3.33 mM, 3.24 mg), L-cysteine (1.66 mM, 0.61 mg), and TBAOH (6.67 mM, 5.19 mg) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. To 150 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to CD analysis (110 μ M) (Figure 21). To 50 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to UV analysis (50 μ M) (Figure 22). The above experiment was repeated in MeOH:water (4:1) and subjected to CD and UV analysis (110 μ M and 50 μ M respectively) (Figures 21 and 22).

[0103] A solution of probe **1** (3.33 mM, 3.24 mg), L-cysteine (1.66 mM, 0.61 mg), and TBAOH (6.67 mM, 5.19 mg) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. To 150 μ L of this solution acetonitrile (2 mL) was added and the mixture was subjected to CD analysis (110 μ M) (Figure 20). The above experiment was repeated with KOH, NaOH, LiOH, Na_2CO_3 , Cs_2CO_3 , K_2CO_3 , and DBU as base and subjected to CD analysis (110 μ M) (Figure 2C).

[0104] A solution of probe **1** (2.5 mM, 2.43 mg), L-cysteine (1.25 mM, 0.45 mg), and varying amount (4, 6, 10, 20, 40 equivalents) of K_2CO_3 in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. To 250 μ L of these solutions acetonitrile (2 mL) was added and the mixtures were subjected to CD analysis (140 μ M) (Figure 23).

[0105] The base screening experiments revealed that superior CD sensing results are obtained using K_2CO_3 , KOH, NaOH, and DBU. Using the base in larger excess did not significantly change the CD response of the probe to cysteine.

Results and Discussion

- 5 [0106] A molecular probe design was favored that would allow fast cysteine detection and generate independent UV and circular dichroism (CD) data for simultaneous determination of a total of three variables: absolute configuration, enantiomeric composition, and overall concentration of the combined enantiomers in aqueous solution. It was envisioned that this could be accomplished by the
- 10 attachment of two chromophores, such as aryl moieties, onto the thiol and amino groups in cysteine. The incorporation of two proximate chromophores would afford a distinct circular dichroism signal that is indicative of the absolute configuration of the major enantiomer (based on the sign of the produced Cotton effect) and also provide the means for er analysis (based on the induced CD amplitude). In contrast with this
- 15 enantioselective sensing mode, a concomitant colorimetric or UV change would be independent of the sample er and therefore suitable for quantification of the total amount of the sensing target. Since modern instruments automatically produce UV and CD spectra at the same time, a dual UV/CD sensing method seemed particularly practical and convenient.
- 20 [0107] In the search for a rugged chiroptical sensing assay that is specific to cysteine a covalent derivatization approach with a chromophoric probe that would generate strong spectroscopic readouts at high wavelengths to avoid interference with other compounds, in particular amino acids, for quantitative analysis at biologically relevant concentrations was favored. Specifically, an inexpensive readily available
- 25 small-molecule probe that combines good solubility and hydrolytic stability for use in aqueous media with sufficient reactivity for fast cysteine detection and chirality recognition even at micromolar levels and at room temperature was sought. The screening of several molecular receptors and binding motifs, including Schiff base formation and metal complexation, led to investigation of the possibility of
- 30 complementary *N*- and *S*-arylation of the amino and thiol functionalities in cysteine using phenyl 2,4-dinitrobenzenesulfonate, **1**, and the 2,4-dinitronaphthalene analogues **2** and **3** (see Figure 1A). The release of fluorescent indicators via nucleophilic

aromatic substitution at a covalently linked dinitrobenzene scaffold has been used for indirect cysteine analysis. (Maeda et al., *Angew. Chem. Int. Ed.* 44:2922–2925 (2005); Jiang et al., *Angew. Chem. Int. Ed.* 46:8445–8448 (2007); Maeda et al., *Angew. Chem. Int. Ed.* 45:1810–1813 (2006); Bouffard et al., *Org. Lett.* 10:37–40 (2008); Shibata et al., *Bioorg. Med. Chem. Lett.* 18:2246–2249 (2008); Ji et al., *J. Org. Chem.* 74:4855–4865 (2009); Athanasiou-Malaki & Koupparis, *Analyst* 112:757–761 (1987) (potentiometric amino acid detection), each of which is hereby incorporated by reference in its entirety.) The three sulfonates were prepared in a single step from the corresponding phenol and naphthol precursors and fully characterized by NMR spectroscopy, crystallography, and combustion analysis (see Examples 2–3).

[0108] Initial evaluation of the cysteine sensing potential of **1–3** revealed strong ultraviolet and CD readouts above 300 nm at approximately 100 μ M concentrations using aqueous acetonitrile as solvent (see, *e.g.*, Figure 1B). The probes are stable to hydrolysis even under basic conditions and the intended double *ipso*-substitution reaction with cysteine was found to generate characteristic CD signals that identify the absolute configuration of the substrate. The sensing of D-cysteine consistently gave two positive Cotton effects, the first in the 400 nm region and a second above 300 nm. The opposite trend was observed with the L-enantiomer. The derivatization with probes **2** and **3** yields the same cysteine analogue and virtually identical CD responses were obtained in these cases as expected (Figures 18 and 19). Although all three probes showed substantial promise, probe **1** was observed to afford the strongest CD amplitude above 400 nm under the same conditions, which is preferable with regard to the sensitivity and ruggedness of the planned quantitative ee sensing applications, *vide infra*.

Example 5—Optimization of the Chiroptical Assay

Mechanistic studies

[0109] *Identification of the sensing product.* The solubility of the sensing product was determined to be 65 mg/mL (ACN:water = 1:1). CD analysis was performed with 1.25 mM cysteine solutions and probe **1**. The CD spectra were collected with a standard sensitivity of 100 mdeg, a data pitch of 0.5 nm, a bandwidth of 1 nm, in a continuous scanning mode with a scanning speed of 500 nm/min and a

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response of 1 s, using a quartz cuvette (1 cm path length). The data were baseline corrected and smoothed using a binomial equation. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm, and a scan rate of 400 nm/s.

5 **[0110]** It was expected that both the thiol and the amino group in cysteine react and consume two equivalents of the probe. The reactivity of the two sites towards the probe was analyzed by reacting 1 equivalent of probe **1** (1.25 mM) with L-cysteine (1.25 mM) and K₂CO₃ (5 mM) in 3 mL of CH₃CN:water (4:1). A second reaction was performed using 2 equivalents of the probe (2.50 mM) with L-cysteine
10 (1.25 mM) and K₂CO₃ (5 mM) in 3 mL of CH₃CN:water (4:1). The resulting mixtures were analyzed by CD and UV spectroscopy at 140 μM and 30 μM, respectively (Figures 25A–B).

[0111] For comparison, *N,S*-bis(2,4-dinitrophenyl)cysteine was synthesized from probe **1** and L-cysteine. CD analysis with this derivative confirmed that cysteine
15 reacts with two equivalents of the probe and forms *N,S*-bis(2,4-dinitrophenyl)cysteine (Figure 26).

[0112] *Reaction analysis.* The reaction between L-cysteine (1.25 mM) and probe **1** (2.50 mM) in the presence of K₂CO₃ (5.0 mM) in 0.85 mL of in CD₃CN:D₂O (4:1) was monitored by ¹H NMR (Figure 27). The reaction was complete within 10
20 minutes under these conditions.

[0113] The cysteine binding (at 1.25 mM) with probe **1** (2.50 mM) in the presence of K₂CO₃ (5.0 mM) in 6 mL of CH₃CN:water (4:1) was monitored using UV-Vis spectroscopy (Figure 24A). Measurements were taken at 10 μM concentration, after dilution of 20 μL reaction mixture aliquots with 2 mL of
25 acetonitrile. The reaction was complete within 10 minutes under these conditions.

[0114] *Racemization analysis.* CD measurements of the sensing mixture using the typical conditions described above (35 μM and K₂CO₃ as base) were taken at 0, 10, 30, 60, and 120 minutes (Figure 28). There was no sign of product racemization.

30 **[0115]** Continued NMR analysis of the sensing mixture under the conditions used in the NMR study shown above (1.25 mM) showed no sign of H/D exchange for the proton H^c attached to the chiral carbon atom in **4** (Figure 29). The integration of the peak for H^c remained constant after 1 hour.

[0116] **Linearity study at higher concentration.** The UV response of probe **1** was measured over a cysteine concentration range from 100 to 1000 μ M (Figure 30A).

[0117] Plotting of the UV absorbance at 355 nm showed a linear sensor
5 response, which indicates the absence of aggregation and self-recognition effects (Figure 30B).

Results and Discussion

[0118] Based on the preliminary results discussed in Example 4, optimization of the chiroptical sensing using **1** as probe continued. To provide evidence that
10 indeed both the amino and the thiol group in Cys undergo *ipso*-substitution, *N,S*-bis(2,4-dinitrophenyl)cysteine, **4**, was prepared and the structure was identified by one- and two-dimensional NMR spectroscopy (Figures 8A–B and Figures 9–13). Comparison of the *in-situ* sensing results using **1** with the CD signature of **4** obtained under typical assay conditions gave superimposable spectra, which confirms the
15 proposed reaction outcome. Accordingly, the addition of more than two equivalents of **1** to a solution containing cysteine does not further change the chiroptical readout and substantially diminished Cotton effects were observed when only one sensor equivalent was employed in the assay protocol. The cysteine sensing also results in the appearance of a new UV absorption band at approximately 350 nm (see, *e.g.*,
20 Figure 2A (left)). The off-on UV response occurs almost immediately upon mixing and reaches a stable maximum within a few minutes (Figures 24A–B). As mentioned above, a nonenantioselective UV readout bears the chance to determine total D/L-cysteine levels, which would complement the *ee* analysis based on the induced CD effects.

25 [0119] In addition, the sensing reaction was monitored by NMR spectroscopy using essentially the same conditions as applied in the chiroptical sensing experiments (Figure 2B). Quantitative conversion of free cysteine to the *N,S*-diarylation product **4** and simultaneous generation of free phenolate occurred within 7 minutes. The reaction is apparently fast and no by-products were detected, which is an important
30 prerequisite for quantitative sensing analysis. Comparison of the NMR spectra obtained with the sensing mixture with the independently prepared compound **4** and phenol in deuterated acetonitrile:water containing four equivalents of K_2CO_3 allowed

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unequivocal reaction analysis (Figure 2B). The attachment of the electron-deficient 3,5-dinitrophenyl rings to the amino and thiol groups results in very strong downfield shifts of the aliphatic cysteine protons H^e and H^f. For example, the proton H^e attached to the chiral carbon center shifted from 3.26 to 4.59 ppm. The *ipso*-substitutions generate two distinguishable 3,5-dinitrophenyl rings. Attachment to the amino moiety causes distinct upfield-shifting of H^b and H^c from 8.49 and 8.13 ppm to 8.14 and 7.05 ppm (labeled as H^{b2} and H^{c2}). As expected, the changes in the NMR shifts are less pronounced for H^{b1} and H^{c1} and for the most downfield-shifted proton H^a. The concomitant cleavage of phenolate from the probe coincides with an upfield shift of the H^d protons from 7.15 and 7.39 ppm to 7.17 and 6.79 ppm, respectively. Altogether, the UV, CD, and NMR experiments demonstrate that quantitative conversion of Cys toward **4** can be accomplished with stoichiometric sensor amounts and the use of large excess of the probe is not necessary. Further studies revealed that aqueous methanol and acetonitrile solutions are suitable solvent mixtures, albeit the latter generates more intense and slightly red-shifted CD and UV signals (Figures 21 and 22). Finally, nine inorganic and organic bases were screened to optimize the sensitivity of the cysteine sensing method. The best results were obtained with NaOH, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), KOH, and K₂CO₃, which was used for all other sensing studies.

Example 6—Cysteine Specificity and Competition Experiments

Experimental Procedures

[0120] **Generally.** Sensing experiments were performed at 1.25 mM cysteine concentration in the presence of other amino acids and competitive biothiols (see Figure 3A). The CD spectra of the resulting mixtures were collected with a standard sensitivity of 100 mdeg, a data pitch of 0.5 nm, a bandwidth of 1 nm, in a continuous scanning mode with a scanning speed of 500 nm/min and a response of 1 s, using a quartz cuvette (1 cm path length). The data were baseline corrected and smoothed using a binomial equation. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm, and a scan rate of 400 nm/s.

[0121] **Selectivity of probe 1 towards cysteine in the presence of other amino acids.** The selectivity of probe **1** towards cysteine in the presence of phenylalanine, alanine, serine, and tyrosine was investigated (Figure 3B). Solutions of probe **1** (2.50

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mM) containing one of the aforementioned L-amino acids (1.25 mM) in the presence of K_2CO_3 (5 mM) in 3 mL of CH_3CN :water (4:1) were stirred for 1 hour. In addition, a solution containing all of the amino acids (1.25 mM) and probe **1** (7.5 mM) in the presence of K_2CO_3 (12.5 mM) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour.

5 The chiroptical responses were analyzed by CD spectroscopy by diluting 200 μ L of each reaction mixture to 2.20 mL (110 μ M) with CH_3CN (Figure 3B (left)). For UV analysis 45 μ L aliquots of each reaction mixture were diluted to 2.045 mL (30 μ M) using CH_3CN (Figure 3B (right)).

[0122] This assay selectively targets cysteine. No CD response was observed
10 with phenylalanine, tyrosine, alanine, and serine. The presence of these amino acids did not alter the chiroptical sensor response to cysteine. Identical Cotton effects were obtained with L-cysteine and with a solution containing L-cysteine in the presence of all other amino acids. (Figure 3B (left)).

[0123] No significant UV response was obtained with phenylalanine, tyrosine,
15 alanine, and serine at 355 nm. The presence of these amino acids did not alter the optical sensor response to cysteine. Identical UV maxima at 355 nm were obtained with L-cysteine and with a solution containing L-cysteine in the presence of all other amino acids. (Figure 3B (right)).

[0124] *Selectivity of probe 1 towards cysteine in the presence of other*
20 *biothiols.* The selectivity of probe **1** towards cysteine in the presence of homocysteine and glutathione was investigated (Figure 3C). Solutions of **1** (2.5 mM) containing one of the L-biothiols (1.25 mM) in the presence of K_2CO_3 (5 mM) in 3 mL of CH_3CN :water (4:1) was stirred for 1 hour. In addition, a solution containing L-cysteine, L-homocysteine, and L-glutathione (1.25 mM) was treated with probe **1** (7.5
25 mM) in the presence of K_2CO_3 (12.5 mM) in 3 mL of CH_3CN :water (4:1) for 1 hour. The chiroptical responses were analyzed by CD spectroscopy by diluting 200 μ L of each reaction mixture to 2.20 mL (110 μ M) with CH_3CN (Figure 3C (left)). For UV analysis aliquots of 45 μ L of each reaction mixture were diluted to 2.045 mL (30 μ M) using CH_3CN (Figure 3C (right)).

30 [0125] This assay selectively targets cysteine. No CD response was observed with homocysteine and glutathione. The presence of these biothiols did not alter the chiroptical sensor response to cysteine. Identical Cotton effects were obtained with

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L-cysteine and with a solution containing L-cysteine in the presence of L-homocysteine and L-glutathione. (Figure 3C (left)).

[0126] The presence of homocysteine and glutathione did not alter the optical sensor response to cysteine significantly. Identical UV maxima at 355 nm were
5 obtained with L-cysteine and with a solution containing L-cysteine in the presence of L-homocysteine and L-glutathione. (Figure 3C (right)).

Results and Discussion

[0127] The irreversibility of the *N,S*-diarylation reaction and the hydrolytic stability of both the probe and *N,S*-bis(2,4-dinitrophenyl)cysteine allow smooth and
10 unambiguous Cys derivatization devoid of interfering by-product formation. The conversion to the chiroptically active product **4** coincides with characteristic and stable UV and CD signals at high wavelengths. Having thus established the basic prerequisites for quantitative stereochemical cysteine sensing, the possibility of substrate specificity in the presence of other substances was evaluated. Interfering
15 stoichiometric amounts of amino acids were tested with phenylalanine, alanine, serine, and tyrosine as representative examples. First, solutions containing probe **1** (2.50 mM) and one of the aforementioned L-amino acids or L-Cys (1.25 mM) in the presence of K₂CO₃ (5 mM) in 3 mL of CH₃CN:water (4:1) were stirred for 1 hour. The mixtures were then further diluted for CD and UV analysis (Figure 3B). All
20 mixtures remained CD silent with the exception of the cysteine solution and similar results were obtained by UV analysis. A solution containing the five amino acids altogether with six equivalents of **1** under otherwise the same conditions was prepared to determine possible interference with the cysteine sensing. Importantly, essentially identical CD and UV responses were measured, which highlights a remarkable
25 specificity of this assay for cysteine.

[0128] The screening of L-homocysteine and L-glutathione, which are common biothiols that often impede the selective targeting of cysteine, was next tested. Glutathione, a cysteine-derived tripeptide, and the non-proteinogenic amino acid homocysteine, which differs only by one additional methylene group from
30 cysteine, display free amine and thiol groups and are therefore very difficult to distinguish from cysteine. When the sensing assay with probe **1** was applied to these biothiols under the conditions described above for the amino acid analysis, a

relatively weak UV response was observed in the 300 nm region but no induction of a CD signal, which is in stark contrast to the cysteine sensing results (Figure 3C). Furthermore, the analysis of a mixture consisting of Cys, H₂Cys, and GSH revealed only a minor contribution of these two biothiols to the UV readout while the CD sensing was not affected at all. The induced Cotton effects obtained with samples containing either L-Cys or a combination of L-Cys, L-H₂Cys, and L-GSH at individual 1.25 mM concentrations in aqueous acetonitrile were virtually identical. The competition experiments thus prove a remarkable specificity of the chiroptical sensing method for cysteine over other amino acids and common biothiols. The optical detection mode is also visible to the naked eye and allows colorimetric cysteine analysis in the presence of amino acids and biothiols (Figure 31).

Example 7—Quantitative Cysteine Analysis at μ M Concentrations

Quantitative cysteine sensing: absolute configuration, enantiomeric excess and total concentration

[0129] *Generally.* The CD spectra of sensing mixtures were collected with a standard sensitivity of 100 mdeg, a data pitch of 0.5 nm, a bandwidth of 1 nm, in a continuous scanning mode with a scanning speed of 500 nm/min and a response of 1 s, using a quartz cuvette (1 cm path length). The data were baseline corrected and smoothed using a binomial equation. UV spectra were collected with an average scanning time of 0.0125 s, a data interval of 5.00 nm, and a scan rate of 400 nm/s.

[0130] *Determination of the concentration of cysteine using probe 1.* The change in the UV absorbance of probe 1 upon cysteine sensing was analyzed (Figures 32A–B). Probe 1 (750 μ M) and L-cysteine in varying concentrations (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 180, 210, 240, 270, 300, and 350 μ M) were dissolved in the presence of K₂CO₃ (1.44 mM) in 2.5 mL of CH₃CN:water (4:1). To 250 μ L of this solution, acetonitrile (2 mL) was added and the mixture was subjected to UV analysis (Figure 32A). The UV absorbance at 355 nm increased as the concentration of cysteine changed from 0 to 350 μ M. Plotting and curve fitting of the UV absorbance at 355 nm against the concentration (μ M) of cysteine gave a linear equation ($y = 0.0023x + 0.0447$) with $R^2 = 0.9946$ (Figure 32B).

[0131] *Determination of the enantiomeric excess of cysteine using probe 1.* A calibration curve was constructed using samples containing cysteine with varying

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enantiomeric composition. Probe **1** (750 μM) and L-cysteine (50 μM) with varying ee's (+100, +80, +60, +40, +20, 0, -20, -40, -60, -80, -100 %ee) were dissolved in the presence of K_2CO_3 (1.44 mM) in 2.5 mL of CH_3CN :water (4:1). After 1.5 hours, CD analysis was carried out using 2 mL of the mixture without further dilution (50 μM) (Figure 33A). The CD amplitudes at 375 and 422 nm were plotted against the enantiomeric excess of cysteine (Figure 33B).

[0132] *Linearity of the CD response versus the concentration of cysteine.*

The change in the CD response of probe **1** upon cysteine recognition was analyzed using varying concentrations of enantiopure L-cysteine (Figure 4A). Probe **1** (750 μM) and L-cysteine in varying concentrations (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 180, 210, 240, 270, 300, and 350 μM) were dissolved in the presence of K_2CO_3 (1.44 mM) in 2.5 mL of CH_3CN :water (4:1). After 1.5 hours, CD analysis was carried out using 2 mL of the mixture without further dilution (Figure 4A (left)). Plotting of the CD amplitudes at 376 and 423 nm of the corresponding reaction mixture against the amount of cysteine showed a linear chiroptical response (Figure 4A (right)).

[0133] *Simultaneous ee and concentration determination.* Six scalemic samples of cysteine were prepared and subjected to simultaneous analysis of the concentration, ee, and absolute configuration using probe **1**. First, a UV spectrum was obtained as described above and the concentration was calculated using regression equation (1) below. Then, a CD spectrum was obtained as described above. This value was then applied in the linear regression equations (2) and (3) to determine the enantiomeric excess. The absolute configuration was determined using the sign of the Cotton effect. The results are shown in Table 1 below.

$$x = \frac{(y - 0.0447)}{0.0023} \quad (\text{Equation 1; } x \text{ in } \mu\text{M})$$

At 375 nm;

$$ee = \frac{\left(\frac{(\text{mdeg} \times 50)}{x} - 0.0797\right)}{0.1032} \quad (\text{Equation 2})$$

At 422 nm;

$$ee = \frac{\left(\frac{(\text{mdeg} \times 50)}{x} + 0.2878\right)}{(-0.1039)} \quad (\text{Equation 3})$$

Table 1. Concentration, ee, and absolute configuration of samples of cysteine determined by the combined UV and CD responses of probe 1.

Cysteine samples			Sensing results				
Abs. config.	Concentration (μM)	% ee	Abs. config.	Concentration (μM)	%ee (375 nm)	%ee (422 nm)	Average %ee
D	100	-40.0	D	105.5	-41.3	-46.8	-44.1
L	50	+80.0	L	54.8	+86.1	+80.6	+83.4
L	75	+20.0	L	79.5	+26.5	+23.9	+25.2
D	90	-11.1	D	95.4	-10.5	-15.0	-12.8
L	18	+44.4	L	21.0	+43.4	+38.8	+41.1
L	40	+25.0	L	42.0	+29.0	+26.0	+27.5

5 **[0134]** *Sensing of cysteine at micromolar concentrations.* A concentration range of 15–30 μM of cysteine has been reported in healthy adult humans and elevated levels of cysteine in the body have been used as indicator for increased risk of Alzheimer's and heart disease. (Oyane et al., *J. Biomed. Mater. Res. A*. 65:188–195 (2003); Brigham et al., 39:1633–1638 (1960), each of which is hereby
10 incorporated by reference in its entirety.) The possibility of cysteine sensing at these physiologically relevant levels was investigated.

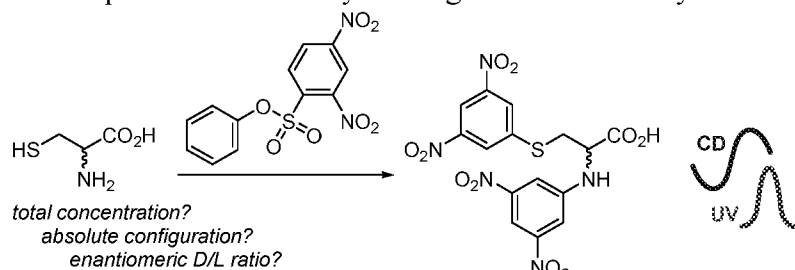
[0135] Probe 1 (150 μM) and L-cysteine (15 μM) with varying ee's (+100, +80, +60, +40, +20, 0, -20, -40, -60, -80, -100 %ee) were dissolved in the presence of K_2CO_3 (60 μM) in 2.5 mL of CH_3CN :water (4:1). After 1.5 hours, CD analysis was
15 carried out using 2 mL of the mixture without further dilution (15 μM) (Figure 4B (left) and Figure 34). The CD amplitudes at 377 and 417 nm were plotted against the enantiomeric excess of cysteine (Figure 4B (right)). The results show that quantitative analysis of micromolar cysteine concentrations is possible.

Results and Discussion

20 **[0136]** Further CD analysis revealed that the chiroptical sensor response increases linearly with the enantiomeric excess and also with the concentration of cysteine (Figure 4A). In addition, the sensing is rugged and practical as recalibration of the UV and CD responses of the probe is not necessary. Having once established the quantitative correlations between the induced UV and CD signals to the

concentration and ee of cysteine, the possibility of sensing at physiologically relevant levels was tested. A concentration range of 15–30 μM of cysteine in healthy adults has been reported and elevated levels of cysteine in the human body have been used as indicator for increased risk of Alzheimer's and heart disease. (Oyane et al., *J. Biomed. Mater. Res. A*, 65:188–195 (2003); Brigham et al., 39:1633–1638 (1960), each of which is hereby incorporated by reference in its entirety.) The possibility of chiroptical cysteine sensing at these concentrations was investigated. Initial CD sensing tests indicated that nonracemic mixtures at 15 μM generate sufficient Cotton effects and can easily be detected and quantified (Figure 4B). This encouraged the ultimate testing of the assay and the probe was applied to six nonracemic samples combining a wide range of cysteine amounts and enantiomeric ratios (Table 2).

Table 2. Comprehensive chirality sensing of nonracemic cysteine samples.



Entry	Cysteine Samples			Chiroptical Sensing Results				
	Abs. Config. ^a	Conc. (μM)	D/L Ratio	Abs. Config. ^a	Conc. (μM)	D/L Ratio (375 nm)	D/L Ratio (422 nm)	Average ^b D/L Ratio
1	D	100	70.0:30.0	D	105.5	70.6:29.4	73.4:26.6	72.1:27.9
2	L	50	10.0:90.0	L	54.8	6.9:93.1	9.7:90.3	8.3:91.7
3	L	75	40.0:60.0	L	79.5	36.6:63.4	38.1:61.9	37.4:62.6
4	D	90	55.5:44.5	D	95.4	55.2:44.8	57.5:42.5	56.4:43.6
5	L	18	27.8:72.2	L	21.0	28.3:71.7	30.6:69.4	29.4:70.6
6	L	40	37.5:62.5	L	42.0	35.5:64.5	37.0:63.0	36.3:63.7

^aMajor enantiomer.

^bBased on the CD sensing at 375 and 422 nm.

[0137] Following the simple mix-and-measure protocol described above, two fast UV and CD measurements allowed simultaneous determination of the absolute configuration of the major cysteine isomer (based on the sign of the induced Cotton effect), the enantiomeric ratio (using the induced CD amplitude), and the overall concentration of the combined enantiomers (from the UV response of the probe) with

good accuracy and without the need to generate a new calibration curve. Of particular interest is the analysis of the 18 μM sample containing the D-form in 27.8% and the L-enantiomer in 72.2% (Table 2, entry 5). Even at this low concentration the sensing results deviated only by a few percent from the actual values. The assay correctly identified L-cysteine as the major enantiomer and the quantitative concentration and enantiomeric determination gave 21 μM and an enantiomeric ratio of 29.4:70.6, respectively. Importantly, samples covering a wide concentration range and drastically different enantiomeric ratios can be analyzed by this method (Table 2, compare entry 1 with entry 5 and entry 2 with entry 4).

10 Example 8—Simulated Body Fluids

Determination of the concentration, enantiomeric excess, and absolute configuration of cysteine in r-SBF (revised simulated body fluids)

[0138] **Generally.** A simulated body fluid (SBF) mimics the ion concentration of the human blood plasma. SBFs have been used for *in-vitro* studies of biologically active compounds. Conventional SBFs have an ion concentration equal to those in the blood plasma, except for Cl^- and HCO_3^- . Revised-SBF (r-SBF) have been introduced to eliminate this discrepancy and were used in this Example. (Fuchs et al., *Mol. Genet. Metab.* 85:168–180 (2005), which is hereby incorporated by reference in its entirety.) The concentration and enantiomeric excess of cysteine at micromolar concentrations in r-SBF were determined using probe 1.

[0139] **Concentration analysis of cysteine samples using probe 1.** The change in the UV absorbance of probe 1 upon cysteine sensing was analyzed (Figures 35A–B). A calibration curve was constructed with samples containing various amounts of cysteine in r-SBF. Probe 1 (750 μM) and L-cysteine in r-SBF in varying concentrations (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 180, 210, 240, 270, 300, and 350 μM) were dissolved in the presence of K_2CO_3 (1.44 mM) in 2.5 mL of CH_3CN :water (4:1). To 250 μL of this solution, acetonitrile (2 mL) was added and the mixture was subjected to UV analysis (Figure 35A). The UV absorbance at 355 nm increased linearly as the concentration of cysteine changed from 0 to 350 μM . Plotting and curve fitting of the UV absorbance at 355 nm against the concentration (μM) of cysteine gave a linear equation ($y = 0.0026x + 0.0634$) with $R^2 = 0.9942$ (Figure 35B).

[0140] Enantiomeric excess analysis of cysteine samples using probe 1.

A calibration curve was constructed using samples containing cysteine in r-SBF with varying enantiomeric composition. Probe 1 (750 μM) and L-cysteine (50 μM) with varying ee (+100, +80, +60, +40, +20, 0, -20, -40, -60, -80, -100 %ee) were dissolved in the presence of K_2CO_3 (1.44 mM) in 2.5 mL of CH_3CN :water (4:1). After 1.5 hours, CD analysis was carried out using 2 mL of the mixture without further dilution (50 μM) (Figure 36A). The CD amplitudes at 365.5 and 409 nm were plotted against the enantiomeric excess of cysteine (Figure 36B).

[0141] Simultaneous ee and concentration determination.

Six scalemic samples of cysteine were prepared and subjected to simultaneous analysis of the concentration, ee, and absolute configuration using probe 1. First, a UV spectrum was obtained as described above, and the concentration was calculated using the regression equation (1) below. Then, a CD spectrum was obtained as described above. This value was then applied in the linear regression equations (2) and (3) to determine the enantiomeric excess. The absolute configuration was determined using the sign of the induced Cotton effect. The results are shown in Table 3 below.

$$x = \frac{(y - 0.0634)}{0.0025} \quad (\text{Equation 1; } x \text{ in } \mu\text{M})$$

At 365.5 nm;

$$ee = \frac{\left(\frac{(\text{mdeg} \times 50)}{x} + 0.1146\right)}{0.1262} \quad (\text{Equation 2})$$

At 409 nm;

$$ee = \frac{\left(\frac{(\text{mdeg} \times 50)}{x} + 0.0075\right)}{(-0.1231)} \quad (\text{Equation 3})$$

Table 3. Concentration, ee, and absolute configuration of samples of cysteine determined by the combined UV and CD responses of probe 1.

Cysteine samples			Sensing results				
Abs. config.	Concentration (μM)	% ee	Abs. config.	Concentration (μM)	%ee (365.5 nm)	%ee (409 nm)	Average %ee
L	130	+53.8	L	129.0	+52.2	+51.0	+51.6

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D	90	-11.1	D	89.6	-11.0	-10.6	-10.8
L	75	+20.0	L	73.1	+17.0	+19.0	+18.0
D	100	-40.0	D	102.9	-38.0	-39.1	-38.6
L	15	+33.3	L	16.6	+23.0	+28.2	+25.6
L	40	+50.0	L	35.1	+44.0	+42.9	+43.5

Results and Discussion

[0142] Because brain fluids and other biological samples containing amino acids in varying nonracemic compositions are not generally available, simulated body fluids (SBFs) were used. SBFs mimic the human blood plasma and have been used for *in-vitro* studies of biologically active compounds. Conventional SBFs have ion concentrations equal to typical values in blood plasma, except for chloride and bicarbonate. A revised-SBF (r-SBF) composition has been introduced to eliminate this discrepancy and was selected to test the ruggedness and usefulness of the Cys sensing assay. (Salemi et al., *Neurol. Sci.* 30:361–364 (2009), which is hereby incorporated by reference in its entirety.) The concentration and enantiomeric ratio of cysteine at micromolar concentrations in r-SBFs containing ions at physiologically relevant levels were determined using probe **1** as described above. The r-SBF was prepared from NaCl, NaHCO₃, Na₂CO₃, KCl, K₂HPO₄, MgCl₂, HEPES, CaCl₂, Na₂SO₄, and by using 1M NaOH and HCl to adjust the pH to 7.40 as described in detail in the literature. The results obtained with six nonracemic samples are shown in Table 4. Again, the absolute configuration of the major isomer together with the ratio and the total concentration of the cysteine enantiomers were determined correctly and with good accuracy. For example, analysis of the sample containing cysteine at 130 μ M with an enantiomeric D/L ratio of 23.1:76.9 gave 129 μ M and 24.2:75.8, respectively (Table 4, entry 1). A comparison of the actual with the experimentally determined data obtained for the second sample (90 μ M, D/L 55.5:44.5) shows almost identical values (89.6 μ M, D/L 55.4:44.6). Overall, significant interference with the cysteine sensing in simulated body fluids is not observed and the sensitivity of this method is not compromised as is evident from the analysis of a 15 μ M sample with a D/L ratio of 33.3:66.7. In this case, the

concentration was determined as 16.6 μM and the enantiomeric ratio as 37.2:62.8 (Table 4, entry 5).

Table 4. Analysis of nonracemic cysteine samples in simulated body fluids.

Entry	Cysteine Samples				Chiroptical Sensing Results		
	Abs. Config. ^a	Conc. (μM)	D/L Ratio		Abs. Config. ^a	Conc. (μM)	D/L Ratio ^b
1	L	130	23.1:76.9		L	129.0	24.2:75.8
2	D	90	55.5:44.5		D	89.6	55.4:44.6
3	L	75	40.0:60.0		L	73.1	41.0:59.0
4	D	100	70.0:30.0		D	102.9	69.3:30.7
5	L	15	33.3:66.7		L	16.6	37.2:62.8
6	L	40	25.0:75.0		L	35.1	28.2:71.8

^aMajor enantiomer.

^bAveraged value based on the CD sensing at 366 and 409 nm.

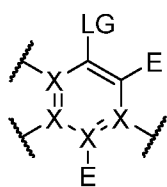
Discussion of Examples 1–8

[0143] Three small-molecule probes were synthesized that are stable to hydrolysis and carry a transferable dinitroaryl moiety for substrate-specific optical sensing of cysteine. The chromophoric probes used in Examples 1–8 attach to the thiol and amino functions of the targeted cysteine within minutes in aqueous solution. The reaction course and outcome with one of the probes was verified by NMR spectroscopy, and by comparison of the UV and circular dichroism readouts observed upon *in situ* cysteine sensing with the chiroptical signals of separately prepared and fully characterized *N,S*-bis(2,4-dinitrophenyl)cysteine. The double *ipso*-substitution results in strong spectroscopic readouts at high wavelengths which provides detailed information about the absolute configuration, enantiomeric ratio, and total concentration of cysteine. The absolute configuration of the major amino acid enantiomer and the D/L ratio can be readily determined from the sign and amplitude, respectively, of the induced CD signals appearing at approximately 370 and 410 nm. In addition, a new UV absorption band at approximately 350 nm appears that is independent of the enantiomeric sample composition and correlated to the overall cysteine concentration. The molecular detection and stereochemical sensing of cysteine is highly substrate-specific and the presence of other amino acids (phenylalanine, alanine, serine, and tyrosine were used as representative examples) or

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biothiols (homocysteine and glutathione) does not interfere with the (chir)optical responses. Finally, quantitative analysis of cysteine in simulated body fluids was demonstrated. The optical assay relies on a practical mix-and-measure protocol and two fast UV and CD measurements that provide comprehensive stereochemical information about the absolute configuration, D/L ratio, and overall amount of cysteine. Samples covering a wide concentration range and drastically different enantiomeric ratios can be analyzed without the need for cumbersome recalibration experiments by this method and accurate values are obtained with aqueous solutions containing cysteine at biologically relevant micromolar levels.

10 **[0144]** It is predicted from Examples 1–8 that other small molecule probes that comprise a chromophore of the formula



(where X, LG, and E are as defined above), would also be useful for analyzing cysteine. Provided the orientation of the leaving group and electron withdrawing group(s) around the (hetero)arylene ring is conserved, it is expected that the probes can tolerate considerable variation elsewhere.

15 **[0145]** Although some embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

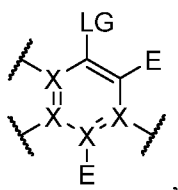
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WHAT IS CLAIMED IS:

1. A compound comprising a chromophore of the formula



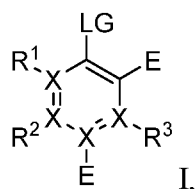
wherein:

each X is independently C or N;

each E is independently hydrogen or an electron withdrawing group selected from the group consisting of $-\text{CF}_3$, $-\text{C}(\text{O})-\text{R}_a$, $-\text{SO}_2-\text{R}_a$, $-\text{CN}$, and $-\text{NO}_2$, with the proviso that at least one E is an electron withdrawing group; and

LG is a leaving group.

2. The compound according to claim 1, wherein the compound is a compound of Formula I:



wherein:

R^1 and R^2 are each independently absent or selected from the group consisting of $-\text{R}_a$, $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; or R^1 and R^2 , together with the carbon atoms to which they are attached, form a cycloalkyl , heterocycloalkyl , aryl , or heteroaryl ;

R^3 is absent or selected from the group consisting of $-\text{R}_a$, $-\text{cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{aryl}$, and $-\text{heteroaryl}$; and

each R_a is independently selected from the group consisting of $-\text{H}$, $-\text{alkyl}$, $-\text{O-alkyl}$, $-\text{N-alkyl}$, $-\text{alkenyl}$, $-\text{O-alkenyl}$, $-\text{N-alkenyl}$, $-\text{alkynyl}$, $-\text{O-alkynyl}$, $-\text{N-alkynyl}$, $-\text{aryl}$, $-\text{O-aryl}$, $-\text{N-aryl}$, $-\text{heteroaryl}$, $-\text{O-heteroaryl}$, $-\text{N-heteroaryl}$, $-\text{cycloalkyl}$, $-\text{O-cycloalkyl}$, $-\text{N-cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{O-heterocycloalkyl}$, and $-\text{N-heterocycloalkyl}$.

3. The compound of claim 2, wherein each X is C.

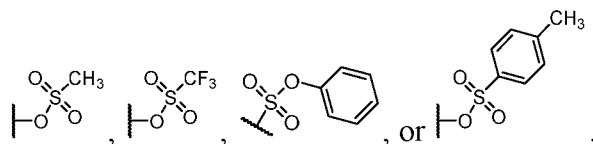
- 47 -

4. The compound of claim 2, wherein each E is an electron withdrawing group.

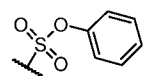
5. The compound of claim 4, wherein each E is $-\text{NO}_2$.

6. The compound of claim 2, wherein LG is a leaving group selected from the group consisting of halogen, $-\text{OR}_b$, $-\text{OC}(\text{O})\text{R}_b$, $-\text{OS}(\text{O})_2\text{R}_b$, $-\text{S}(\text{O})_2-\text{O}-\text{R}_b$, $-\text{N}_2^+$, $-\text{N}^+(\text{R}_b)_3$, $-\text{S}^+(\text{R}_b)_2$, and $-\text{P}^+(\text{R}_b)_3$; wherein each R_b is independently selected from the group consisting of hydrogen, $-\text{alkyl}$, $-\text{O-alkyl}$, $-\text{N-alkyl}$, $-\text{alkenyl}$, $-\text{O-alkenyl}$, $-\text{N-alkenyl}$, $-\text{alkynyl}$, $-\text{O-alkynyl}$, $-\text{N-alkynyl}$, $-\text{perfluoroalkyl}$, $-\text{perfluoroalkenyl}$, $-\text{perfluoroalkynyl}$, $-\text{aryl}$, $-\text{O-aryl}$, $-\text{N-aryl}$, $-\text{heteroaryl}$, $-\text{O-heteroaryl}$, $-\text{N-heteroaryl}$, $-\text{cycloalkyl}$, $-\text{O-cycloalkyl}$, $-\text{N-cycloalkyl}$, $-\text{heterocycloalkyl}$, $-\text{O-heterocycloalkyl}$, and $-\text{N-heterocycloalkyl}$.

7. The compound of claim 6, wherein the leaving group is



8. The compound of claim 7, wherein the leaving group is



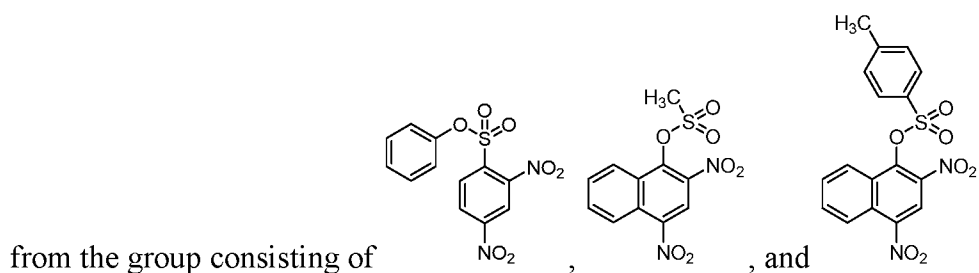
9. The compound of claim 2, wherein R_1 and R_2 , together with the carbon atoms to which they are attached, form a cycloalkyl, heterocycloalkyl, aryl, or heteroaryl.

10. The compound of claim 9, wherein R_1 and R_2 , together with the carbon atoms to which they are attached, form a naphthalene ring.

11. The compound of claim 2, wherein R^1 , R^2 , and R^3 are each hydrogen.

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12. The compound of claim 2, wherein the compound is selected



13. An analytical method comprising:

contacting a sample potentially containing D-cysteine, L-cysteine, or a mixture thereof with a probe, wherein the probe is a compound of claim 1 and said contacting is carried out under conditions effective to result in double *ipso*-substitution of any cysteine present in the sample; and

determining, based on any double *ipso*-substituted cysteine that forms, the absolute configuration of D/L-cysteine in the sample, and/or the enantiomeric composition of cysteine in the sample, and/or the concentration of total cysteine in the sample.

14. The analytical method of claim 13, further comprising:

providing the sample; and

providing the probe.

15. The analytical method of claim 13, wherein the sample is an aqueous solution.

16. The analytical method of claim 13, wherein the sample is a biological sample from an animal.

17. The analytical method of claim 16, wherein the biological sample is selected from the group consisting of blood, plasma, brain extracellular fluid, cerebrospinal fluid, tissue, cells, cell extracts, cell lysates, serum, semen, amniotic fluid, sputum, urine, feces, bodily fluids, bodily secretions, bodily excretions, circulating tumor cells, tumor, tumor biopsy, and exosomes.

18. The analytical method of claim 16, wherein the animal is a vertebrate.

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19. The analytical method of claim 16, wherein the animal is selected from the group consisting of primates, felines, canines, equines, camelids, bovines, caprines, ovines, swine, rabbits, rodents, reptiles, fish, and amphibians.

20. The analytical method of claim 19, wherein the animal is a human subject.

21. The analytical method of claim 16, wherein the animal is a patient having or suspected of having or at risk of having a disorder mediated by free form cysteine.

22. The analytical method of claim 21, wherein the disorder is selected from the group consisting of neurodegenerative disease, amyotrophic lateral sclerosis, frontotemporal lobar degeneration, Parkinson's disease, Huntington's disease, mild cognitive impairment, Alzheimer's disease, diseases associated with TPD-43 proteinopathy, metabolic syndrome, central adiposity, hyperglycemia, hypertension, dyslipidemia, insulin resistance, diabetes, obstructive sleep apnea, irritable bowel disease/inflammatory bowel disease, diseases associated with skeletal wasting or muscle fatigue, HIV/SIV infection, cancer, major injuries, sepsis, Crohn's disease, ulcerative colitis, and chronic fatigue syndrome.

23. The analytical method of claim 13, wherein the absolute configuration of D/L-cysteine in the sample is determined.

24. The analytical method of claim 23, wherein the absolute configuration is determined using circular dichroism spectroscopy, optical rotatory dispersion, or polarimetry.

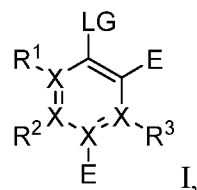
25. The analytical method of claim 13, wherein the enantiomeric composition of cysteine in the sample is determined.

26. The analytical method of claim 25, wherein the enantiomeric composition is determined using circular dichroism spectroscopy, optical rotatory dispersion, or polarimetry.

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27. The analytical method of claim 26, wherein the enantiomeric composition is determined using circular dichroism spectroscopy.
28. The analytical method of claim 13, wherein the concentration of total cysteine in the sample is determined.
29. The analytical method of claim 28, wherein the concentration is determined using UV/Vis spectroscopy, fluorescence spectroscopy, and/or other spectroscopic techniques.
30. The analytical method of claim 29, wherein the concentration is determined using UV/Vis spectroscopy.
31. The analytical method of claim 29, wherein the concentration is determined using fluorescence spectroscopy.
32. The analytical method of claim 13, wherein the absolute configuration of D/L-cysteine in the sample and the concentration of total cysteine in the sample are determined.
33. The analytical method of claim 13, wherein the enantiomeric composition of cysteine in the sample and the concentration of total cysteine in the sample are determined.
34. The analytical method of claim 13, wherein the absolute configuration of D/L-cysteine in the sample, the enantiomeric composition of cysteine in the sample, and the concentration of total cysteine in the sample are all determined.
35. The analytical method of claim 13, wherein the absolute configuration of D/L-cysteine in the sample, the enantiomeric composition of cysteine in the sample, and the concentration of total cysteine in the sample are all determined concomitantly.
36. The analytical method of claim 13, wherein the total cysteine is present in the sample in the micromolar range.

37. The analytical method according to claim 13, wherein the probe is a compound of Formula I:



wherein:

R^1 and R^2 are each independently absent or selected from the group consisting of $-R_a$, $-cycloalkyl$, $-heterocycloalkyl$, $-aryl$, and $-heteroaryl$; or R^1 and R^2 , together with the carbon atoms to which they are attached, form a $cycloalkyl$, $heterocycloalkyl$, $aryl$, or $heteroaryl$;

R^3 is absent or selected from the group consisting of $-R_a$, $-cycloalkyl$, $-heterocycloalkyl$, $-aryl$, and $-heteroaryl$; and

each R_a is independently selected from the group consisting of $-H$, $-alkyl$, $-O-alkyl$, $-N-alkyl$, $-alkenyl$, $-O-alkenyl$, $-N-alkenyl$, $-alkynyl$, $-O-alkynyl$, $-N-alkynyl$, $-aryl$, $-O-aryl$, $-N-aryl$, $-heteroaryl$, $-O-heteroaryl$, $-N-heteroaryl$, $-cycloalkyl$, $-O-cycloalkyl$, $-N-cycloalkyl$, $-heterocycloalkyl$, $-O-heterocycloalkyl$, and $-N-heterocycloalkyl$.

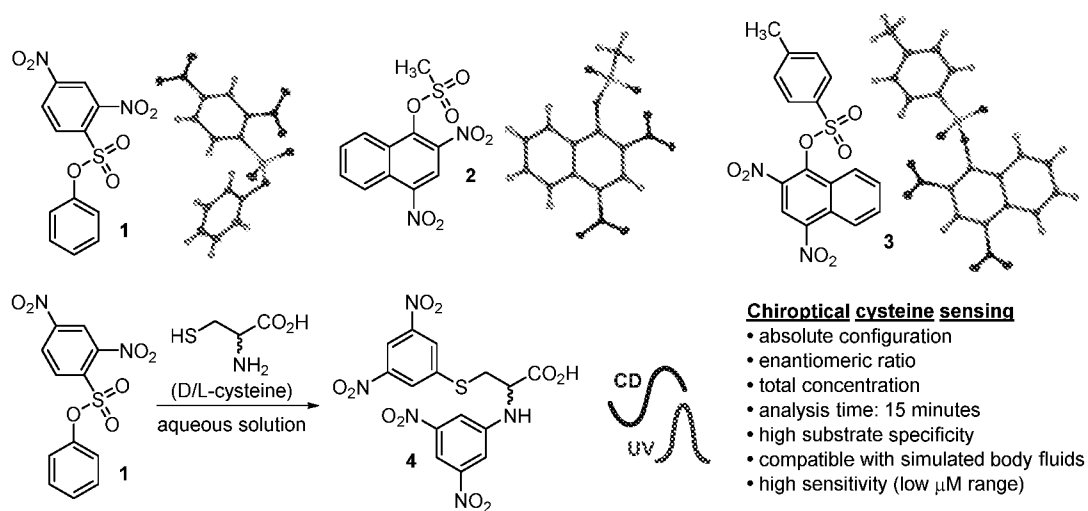


Figure 1A

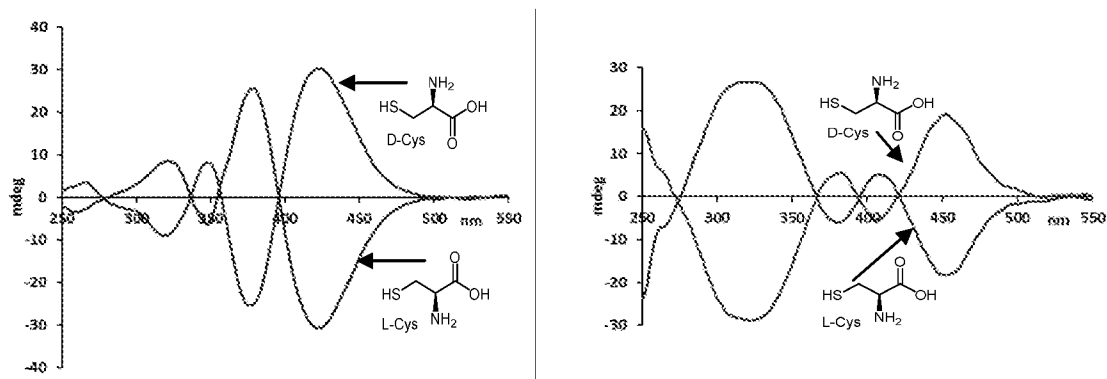


Figure 1B

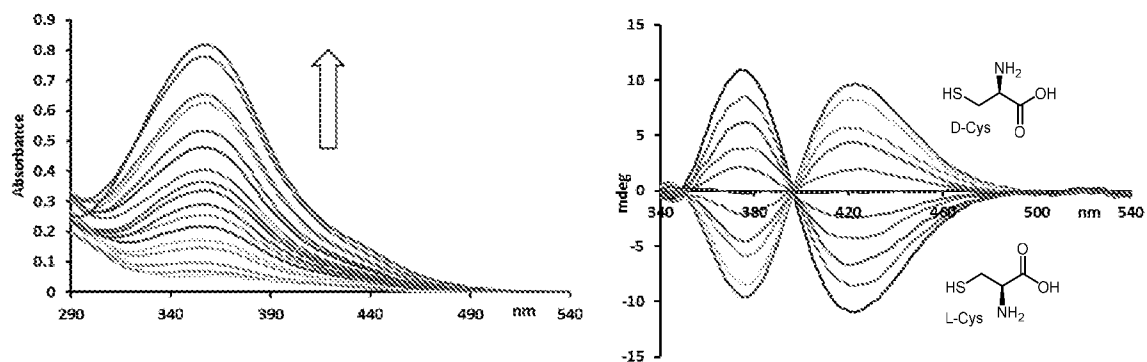


Figure 2A

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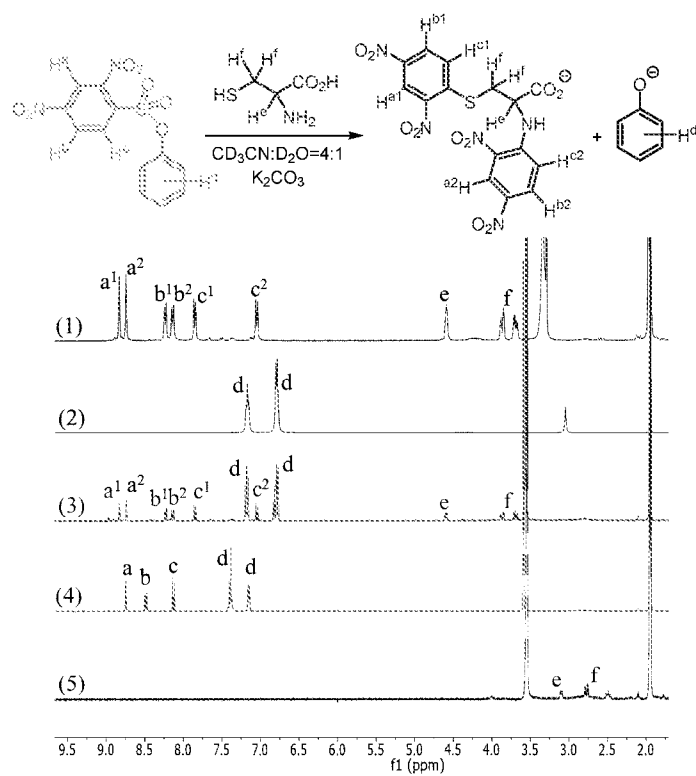


Figure 2B

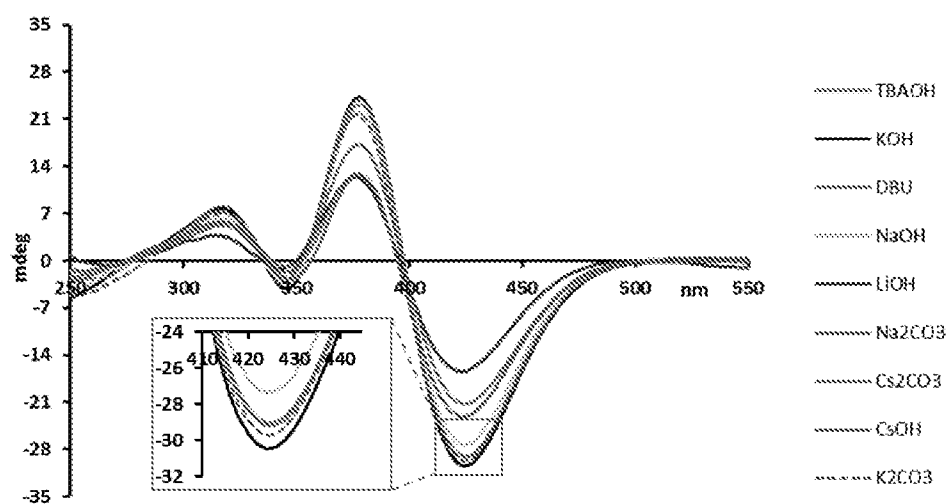


Figure 2C

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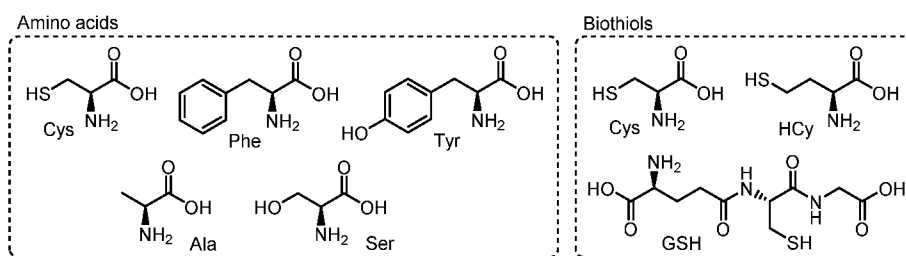


Figure 3A

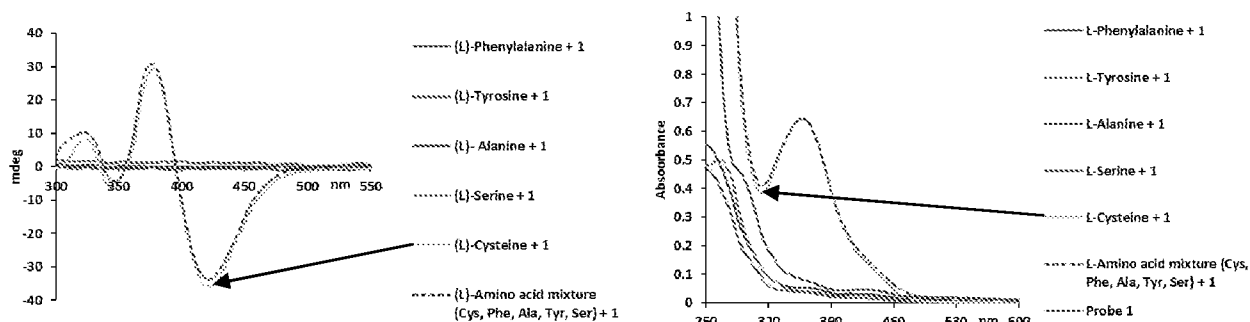


Figure 3B

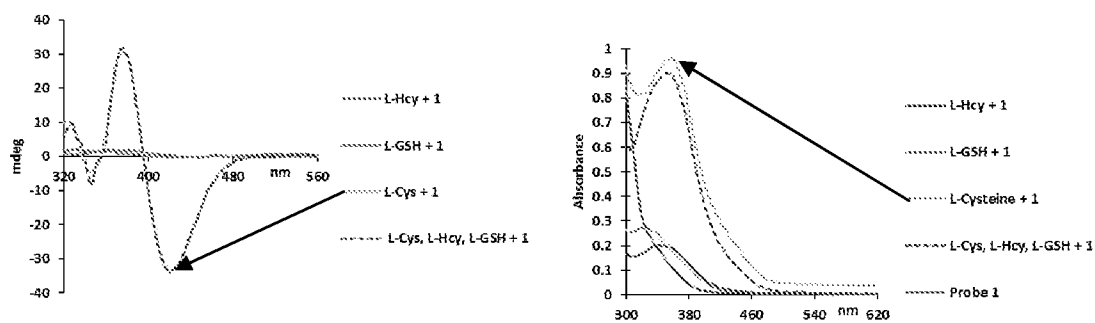


Figure 3C

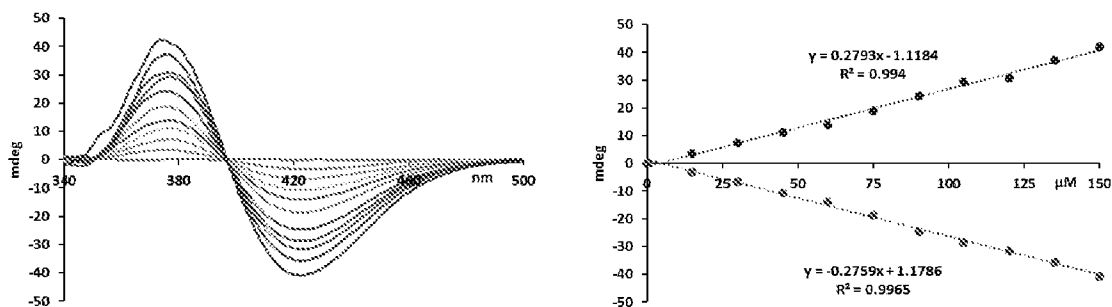


Figure 4A

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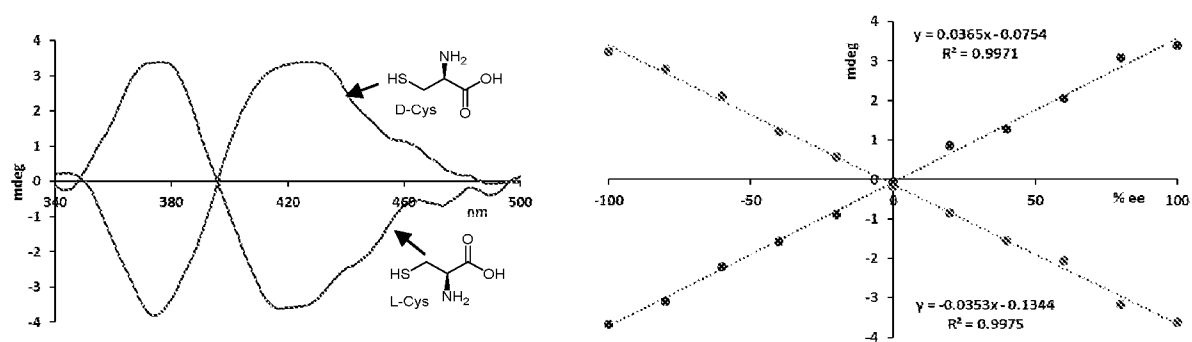


Figure 4B

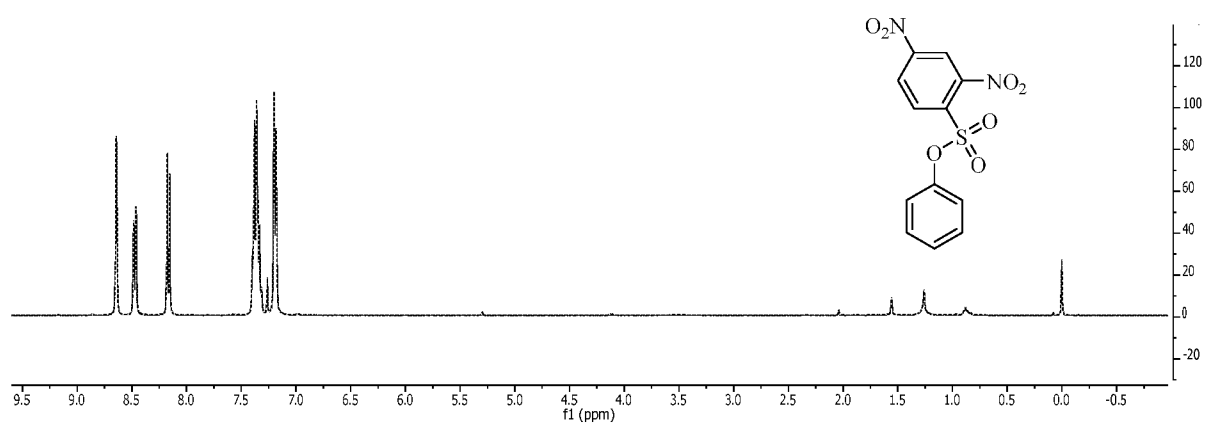


Figure 5A

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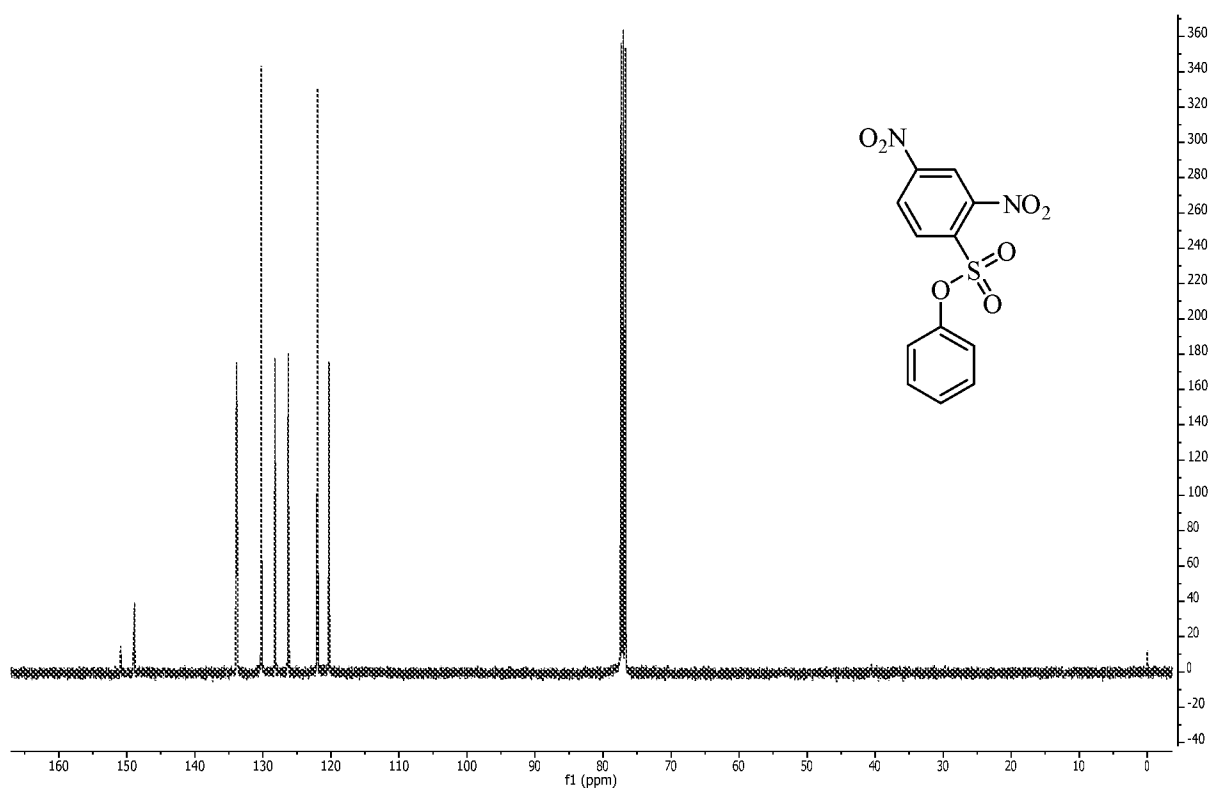


Figure 5B

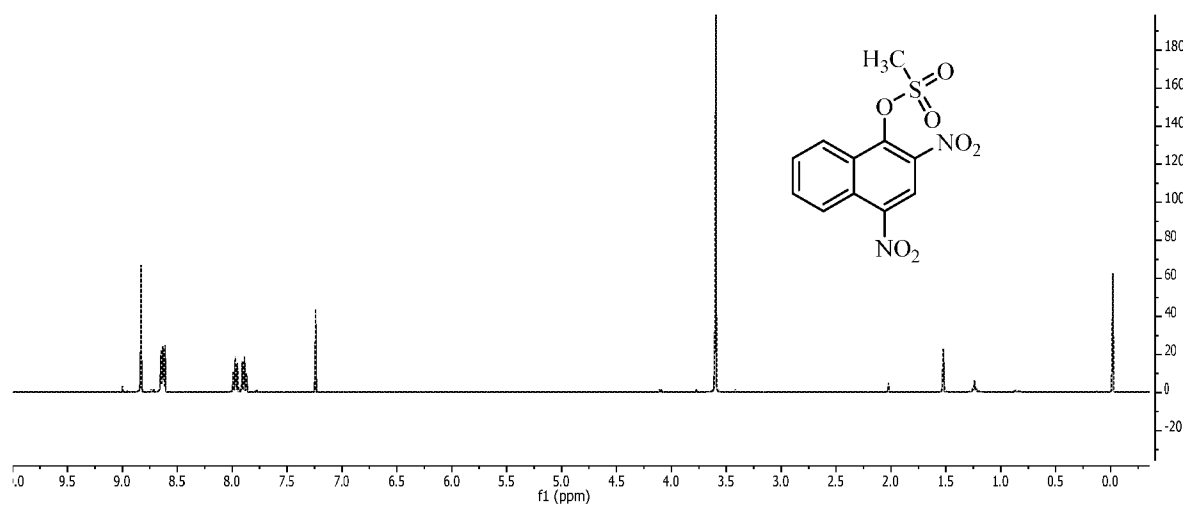


Figure 6A

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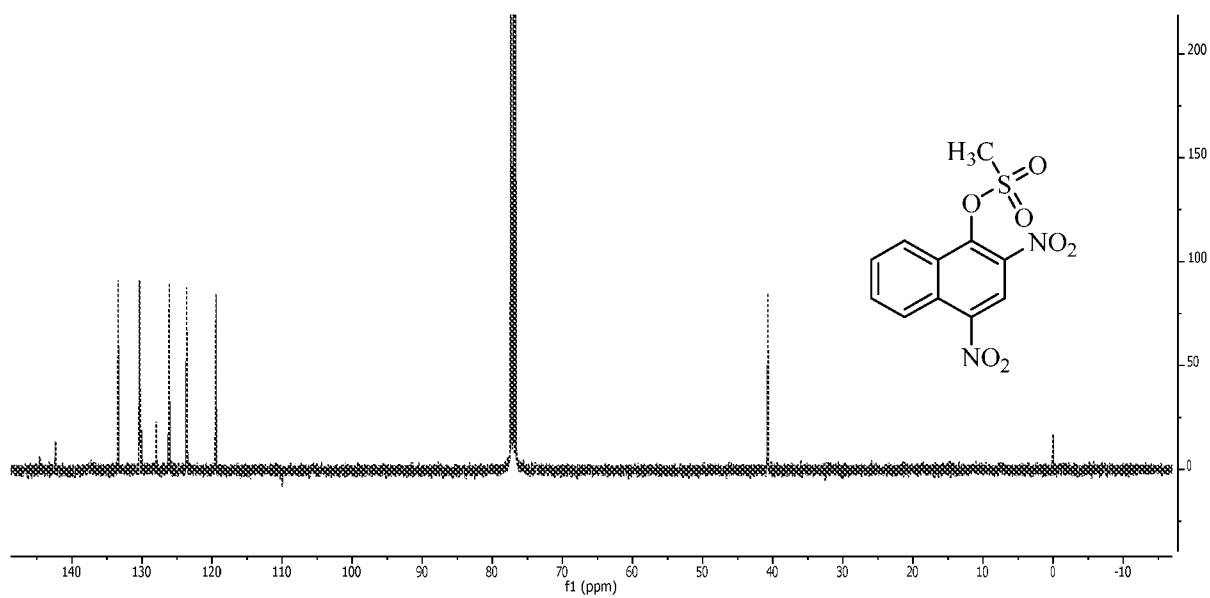


Figure 6B

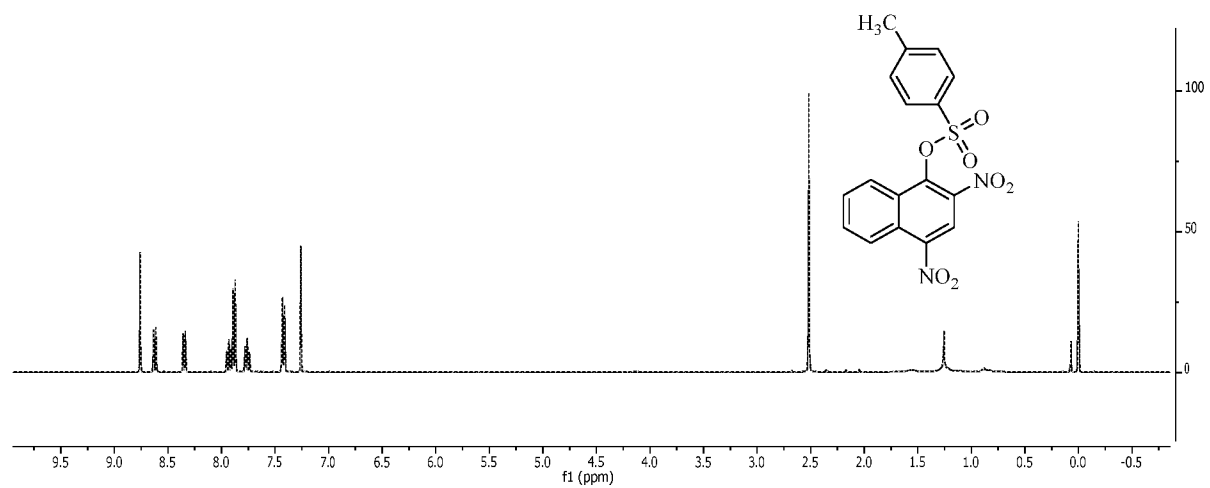


Figure 7A

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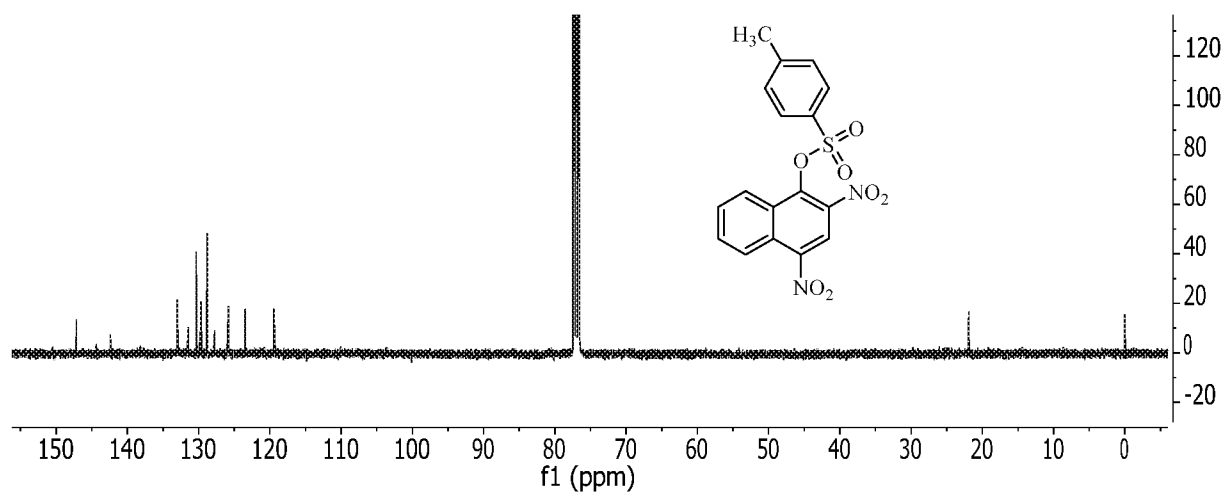


Figure 7B

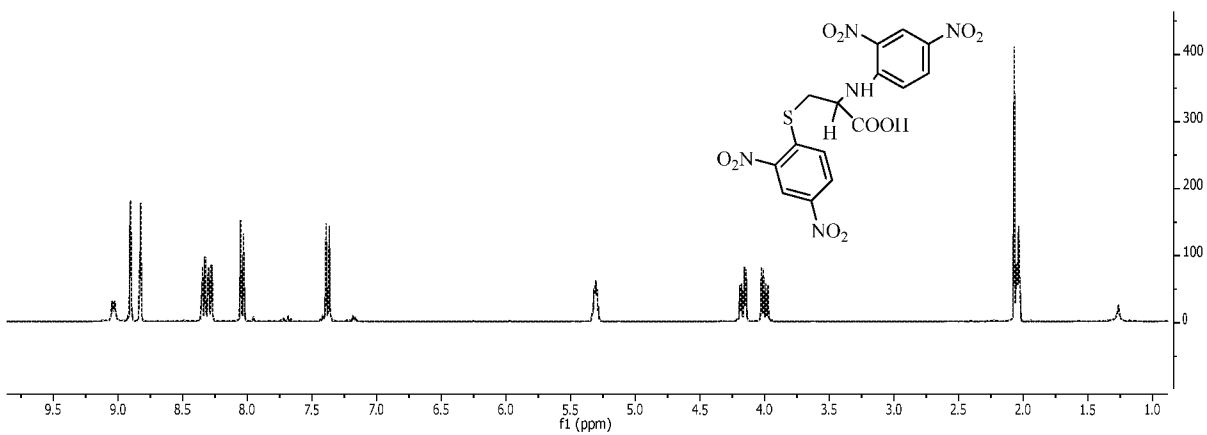


Figure 8A

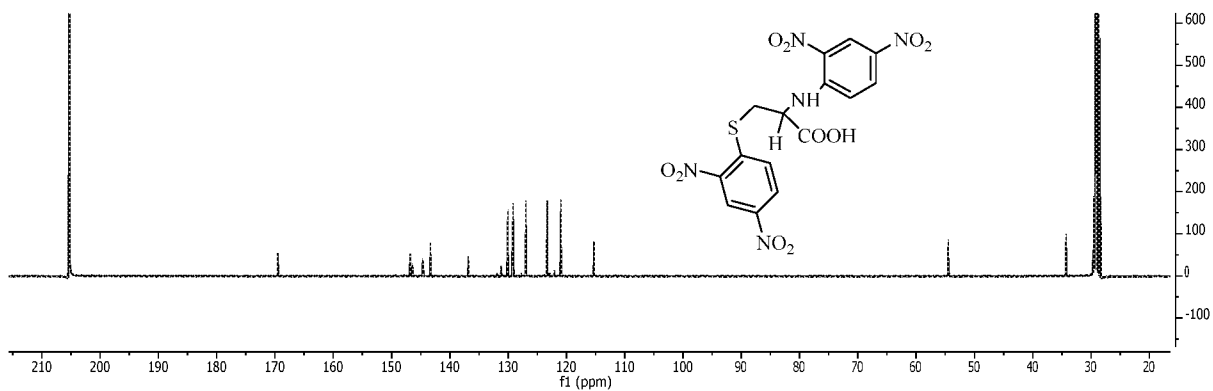


Figure 8B

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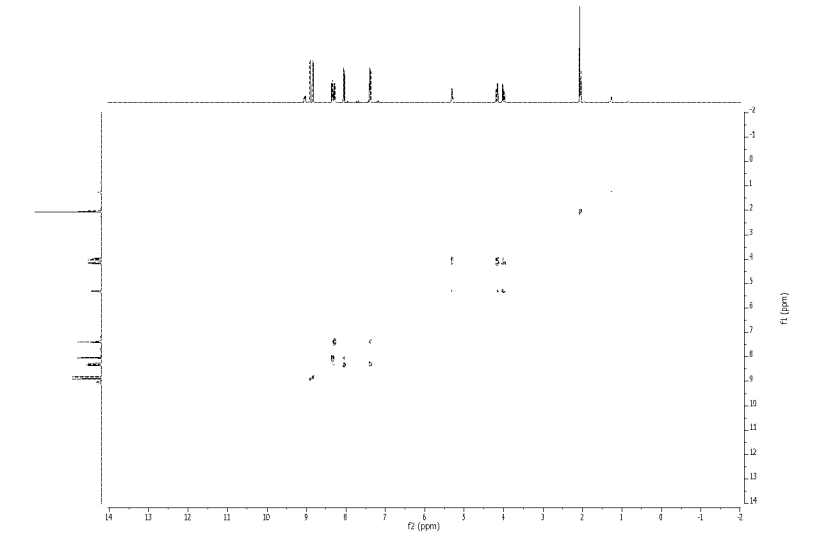


Figure 9

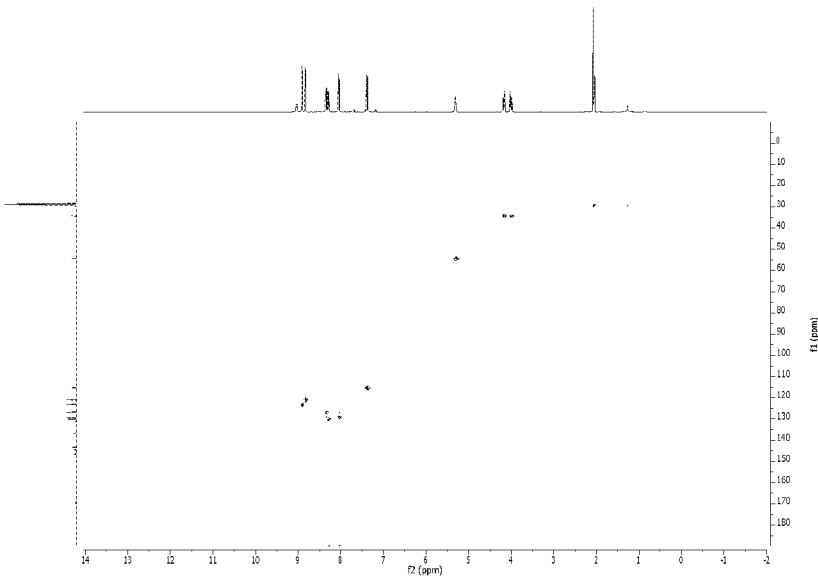


Figure 10

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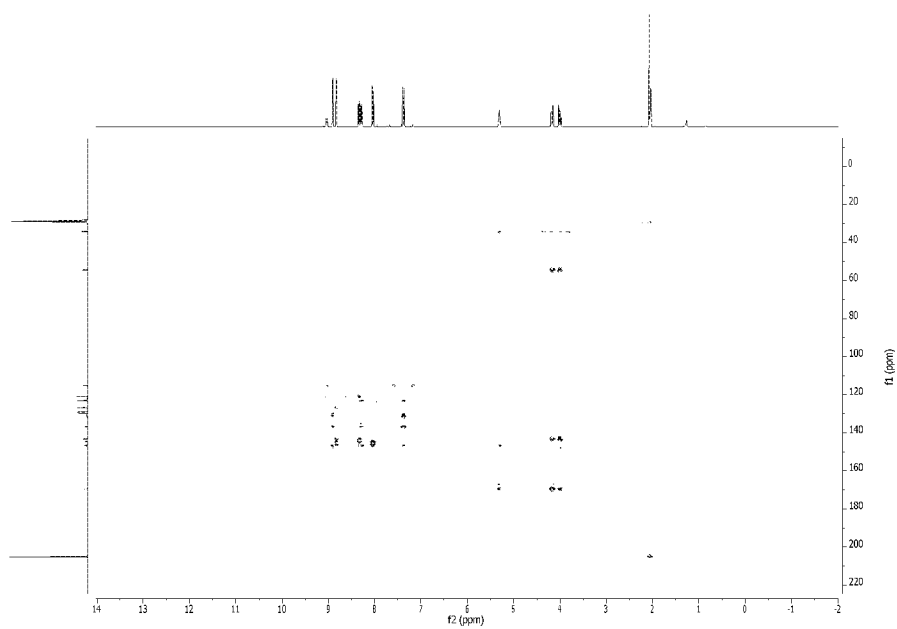


Figure 11

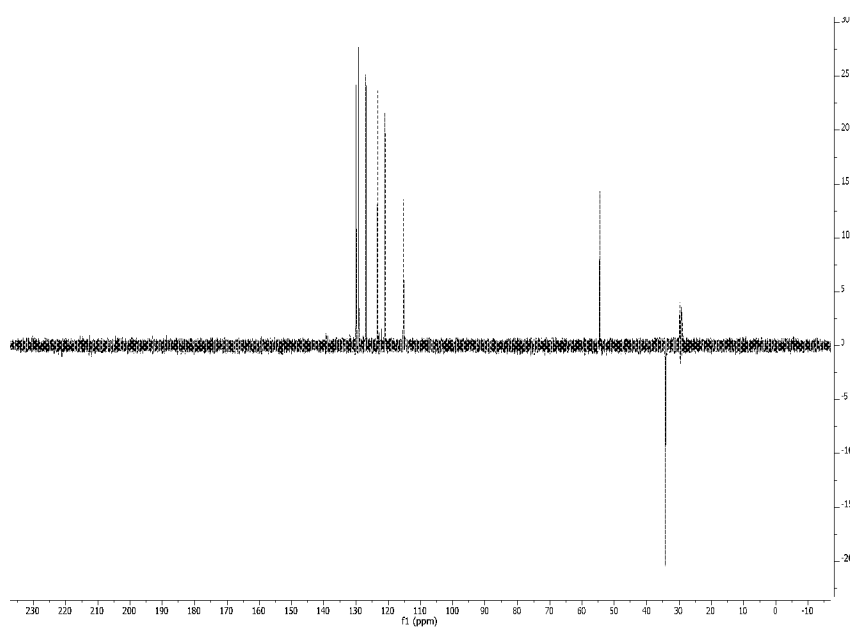


Figure 12

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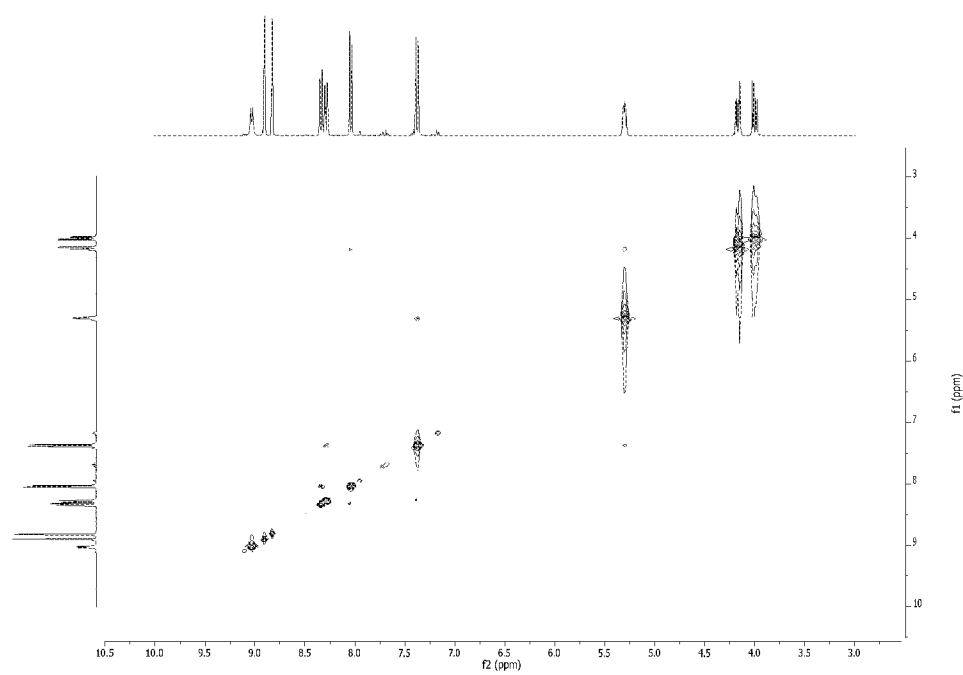


Figure 13

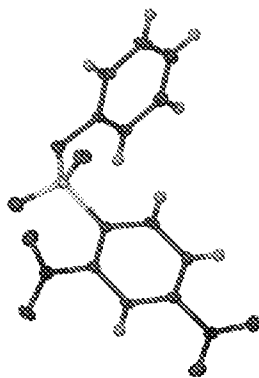


Figure 14

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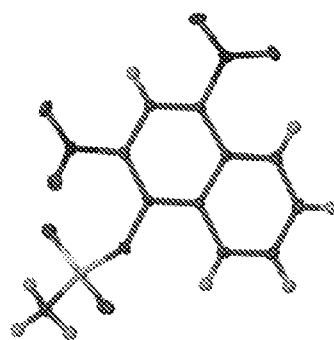


Figure 15

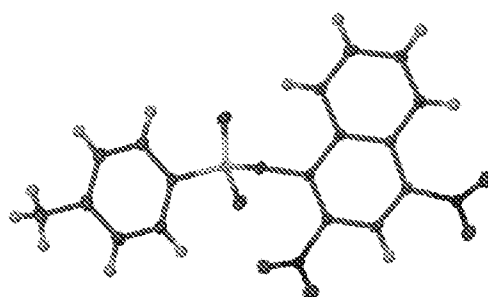


Figure 16

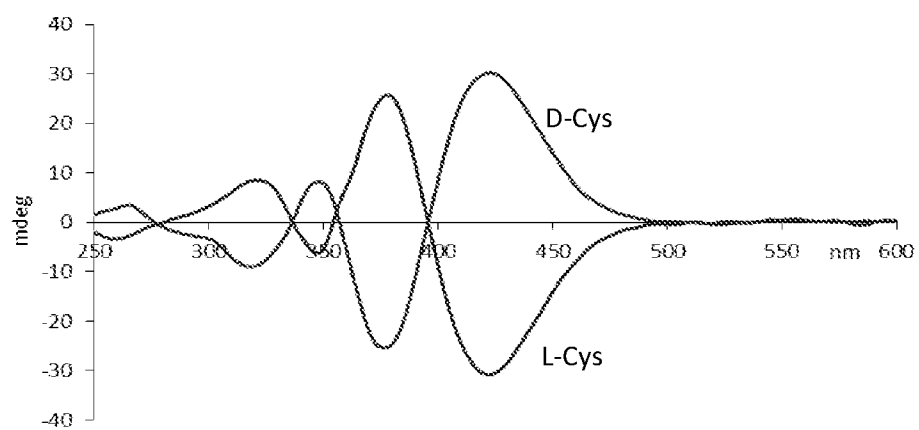


Figure 17

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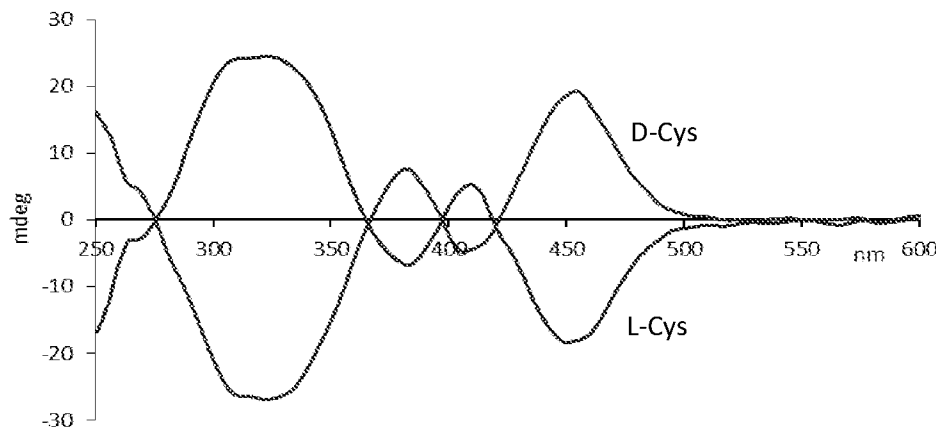


Figure 18

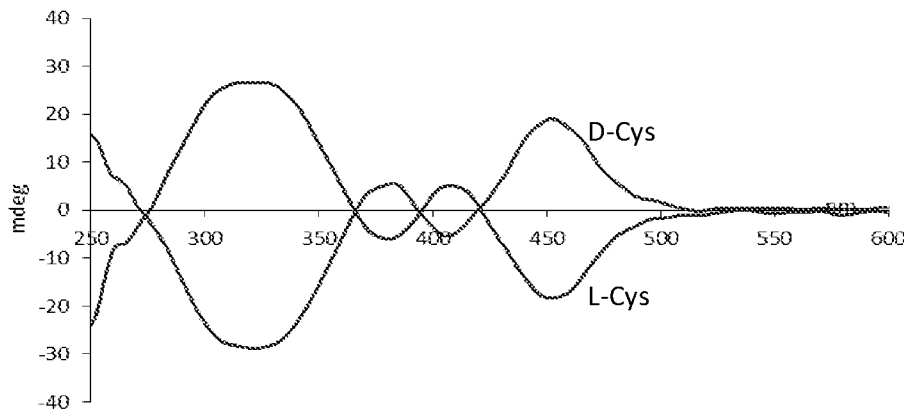


Figure 19

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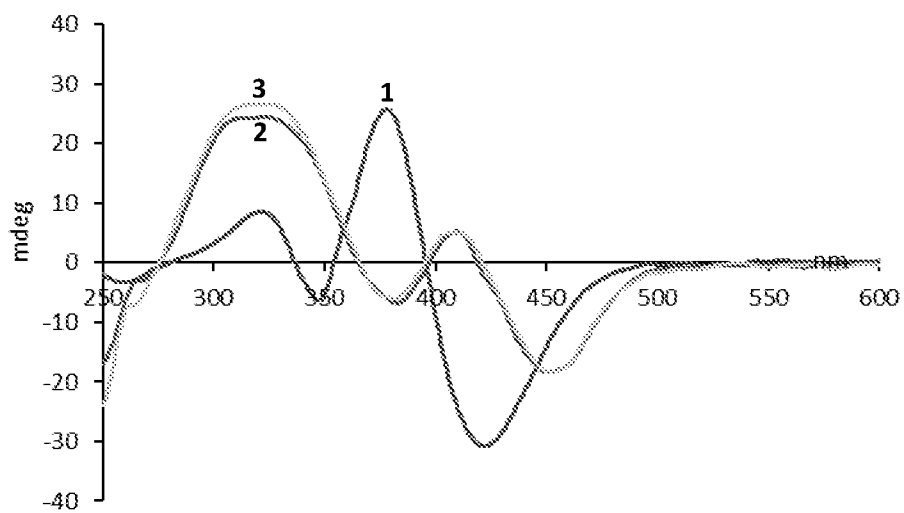


Figure 20

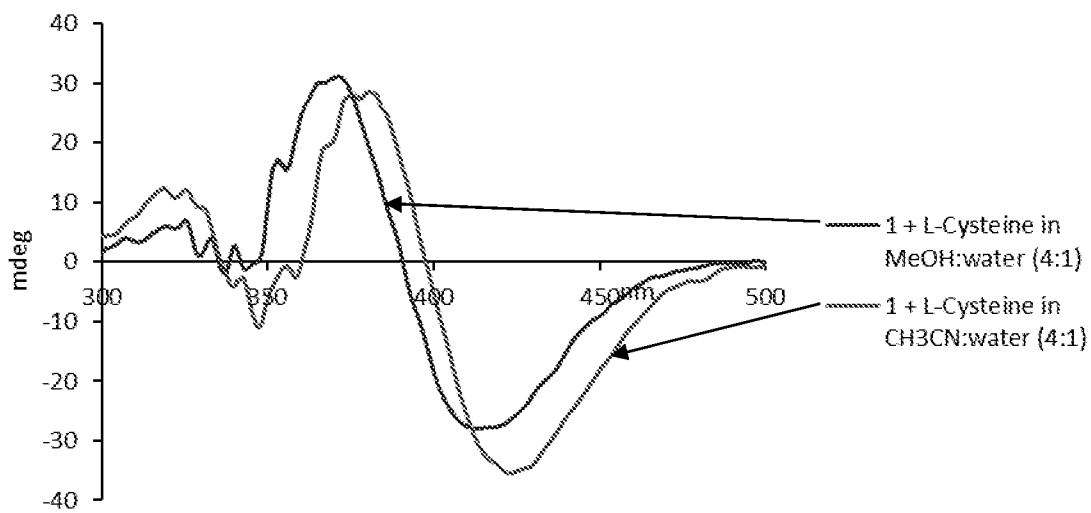


Figure 21

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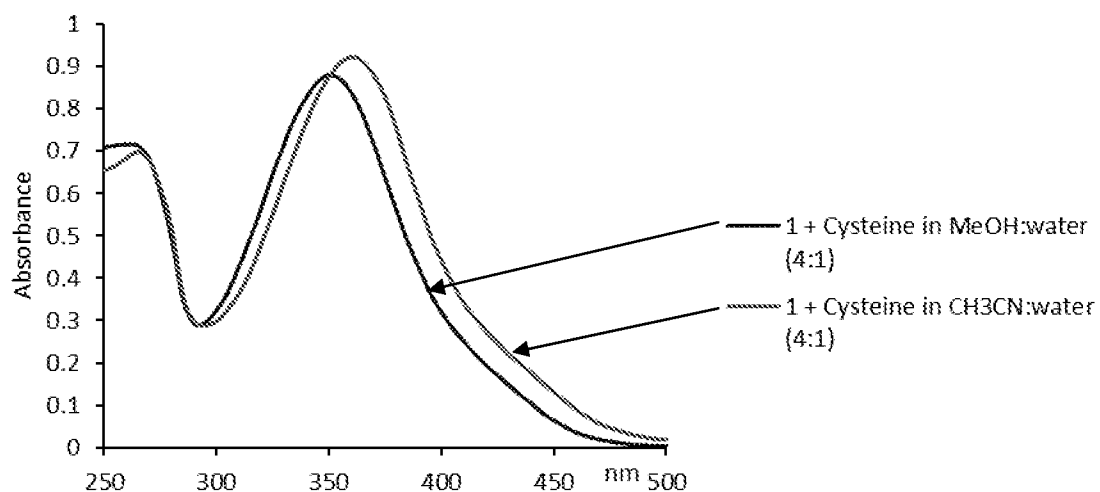


Figure 22

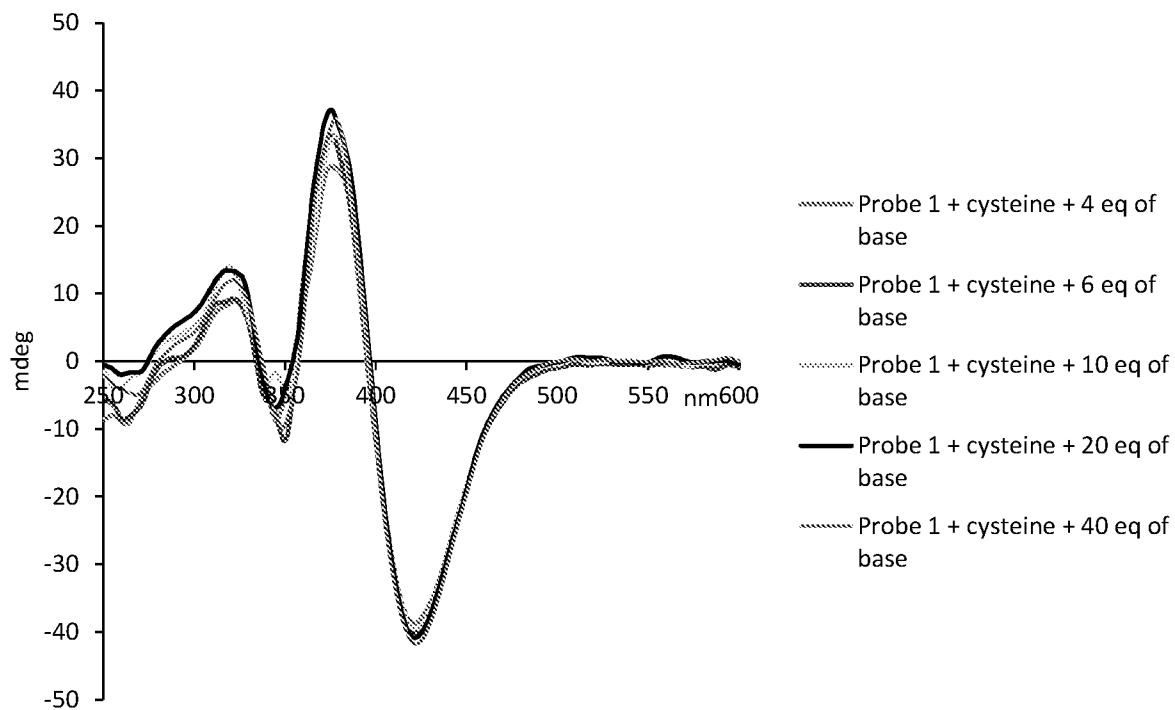


Figure 23

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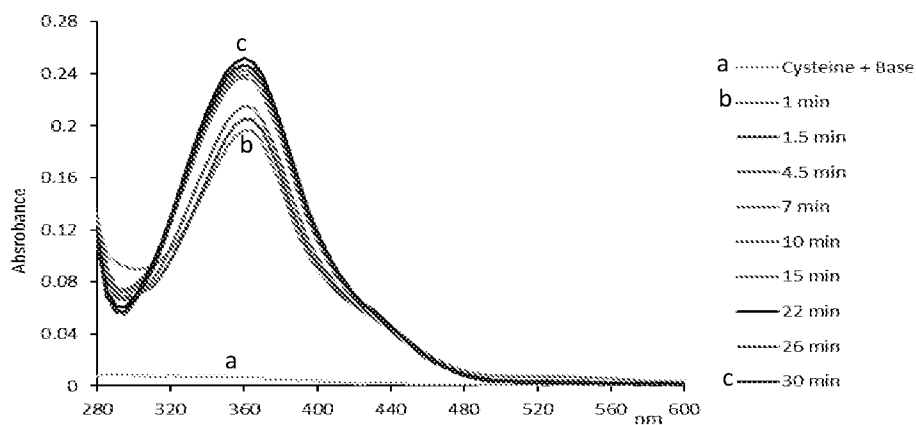


Figure 24A

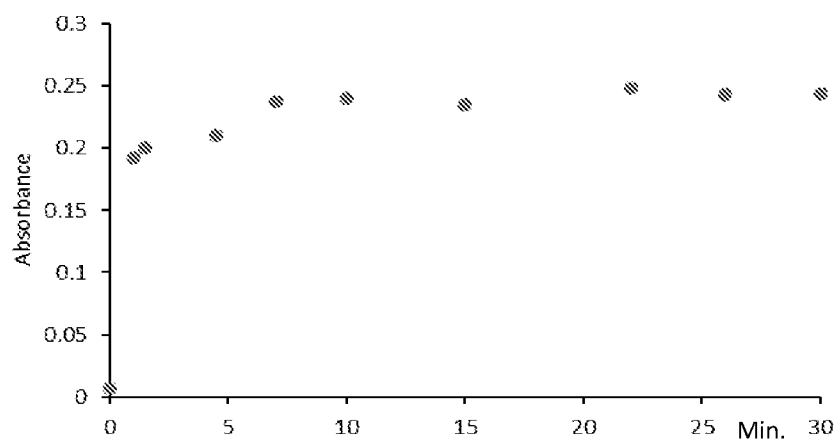


Figure 24B

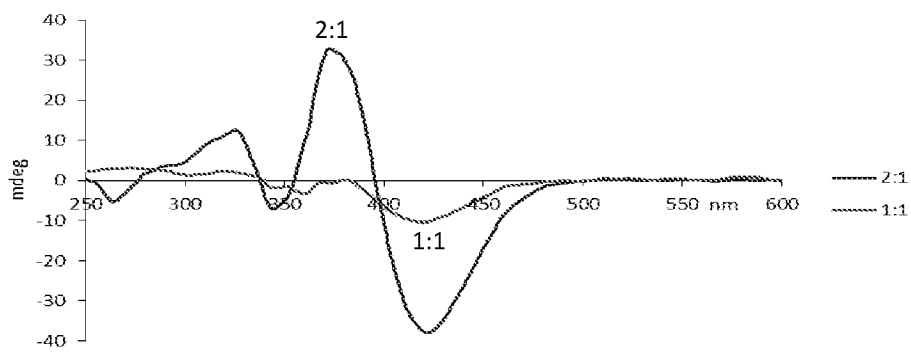


Figure 25A

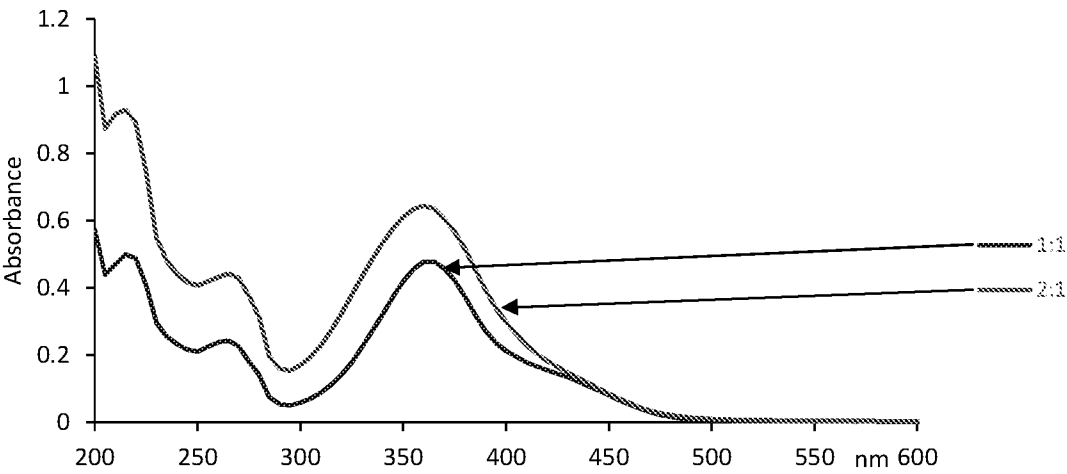


Figure 25B

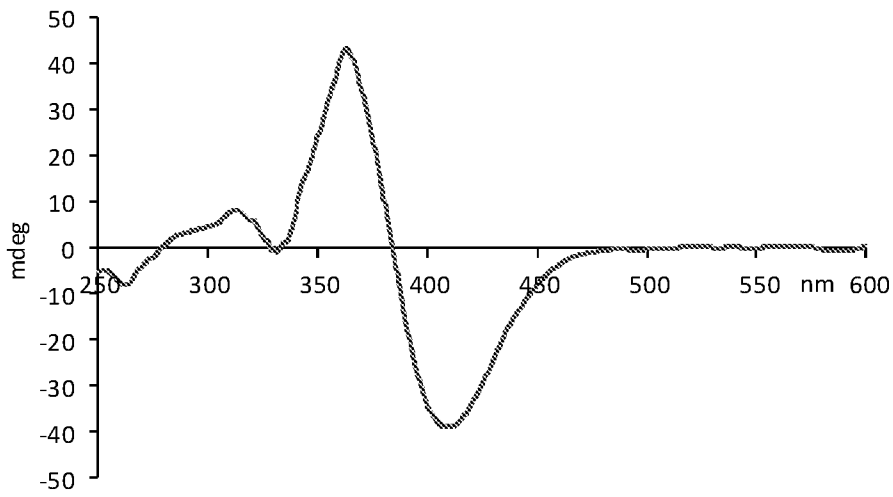


Figure 26

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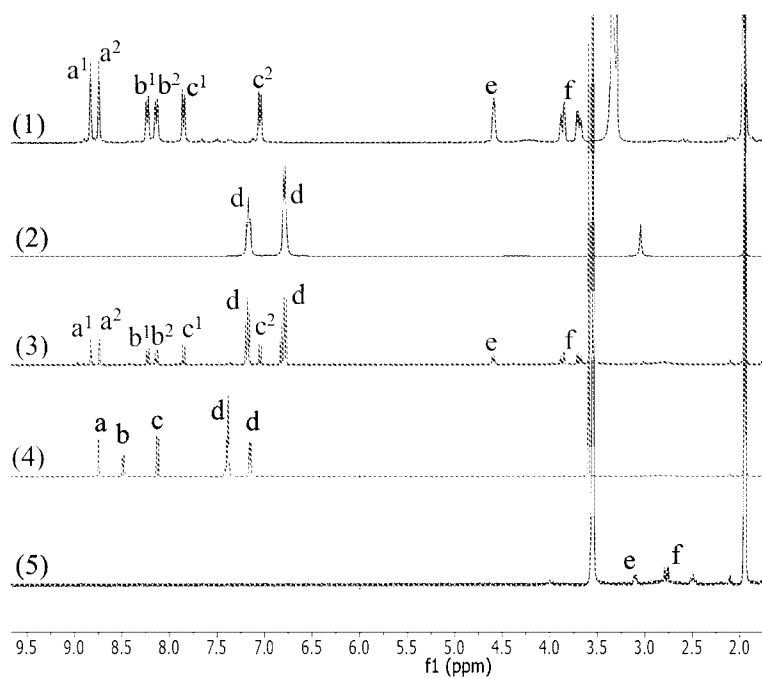


Figure 27

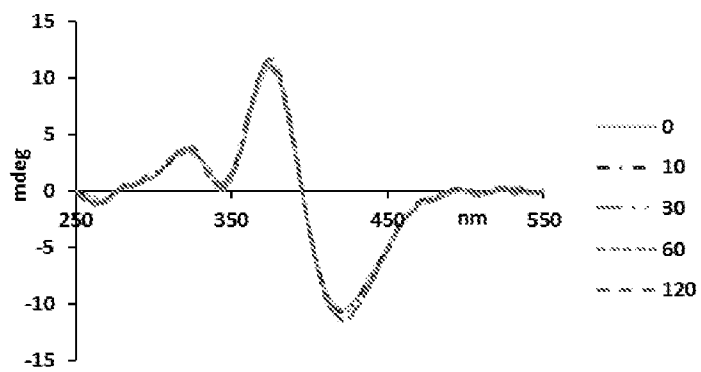


Figure 28

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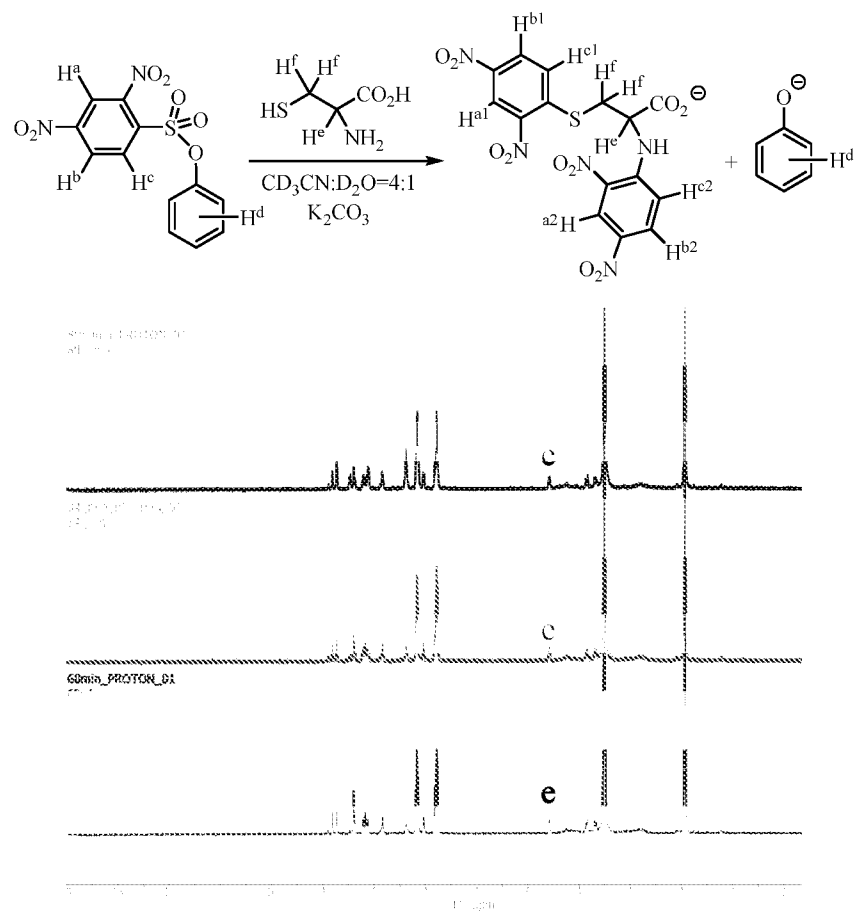


Figure 29

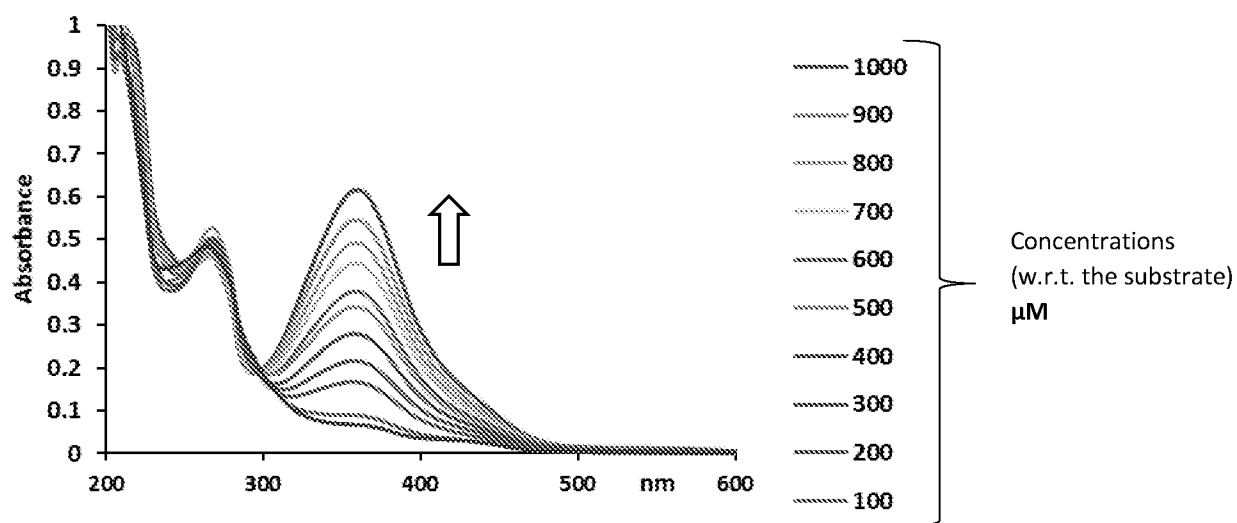


Figure 30A

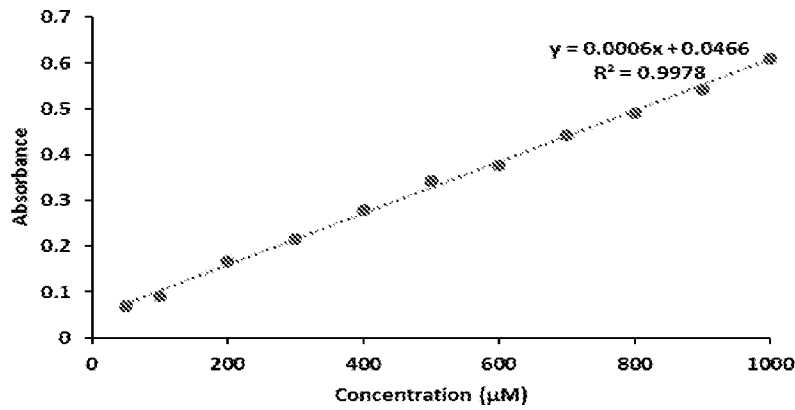


Figure 30B

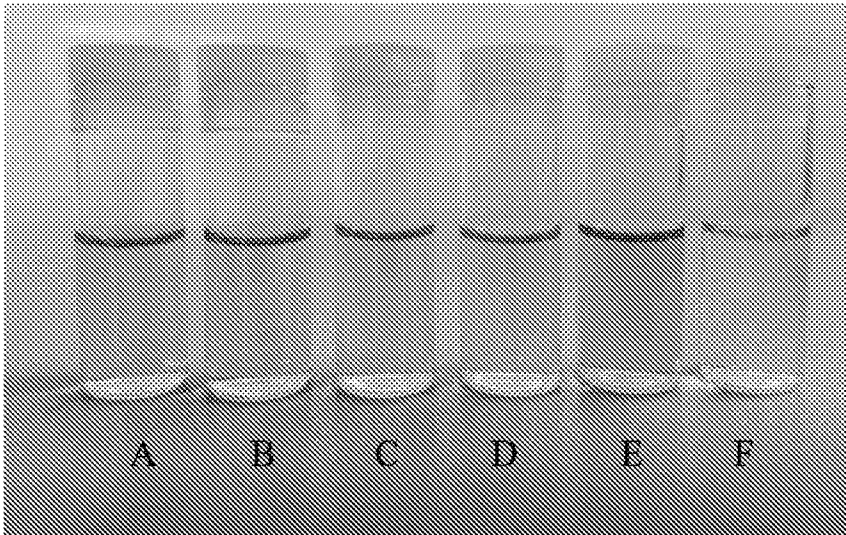


Figure 31

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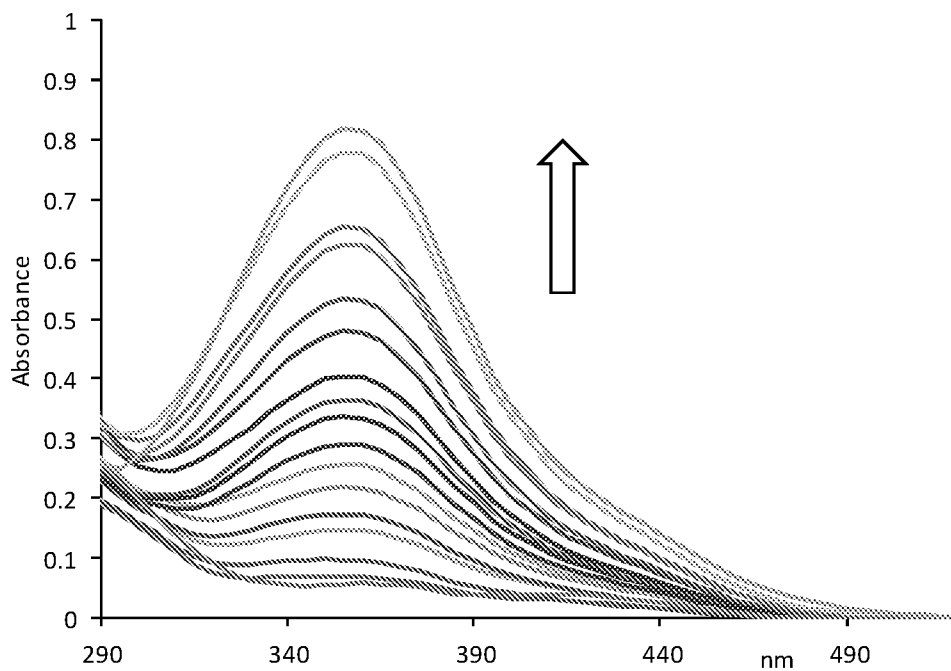


Figure 32A

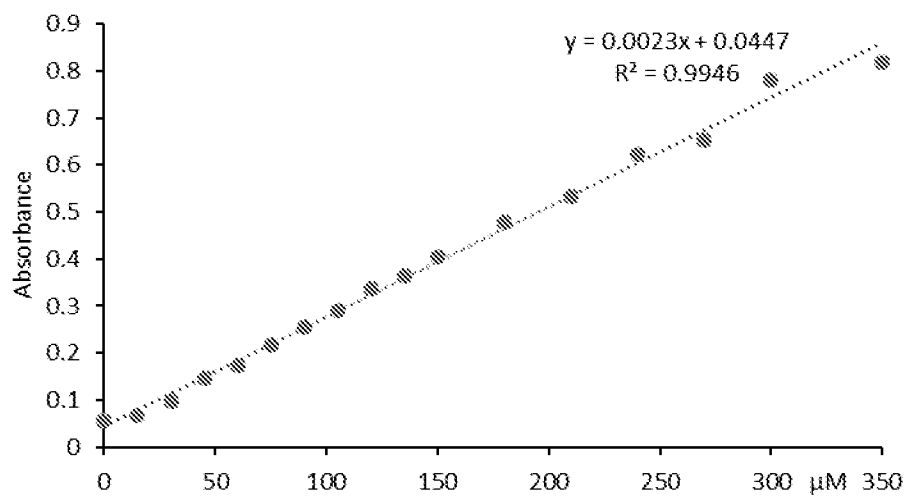


Figure 32B

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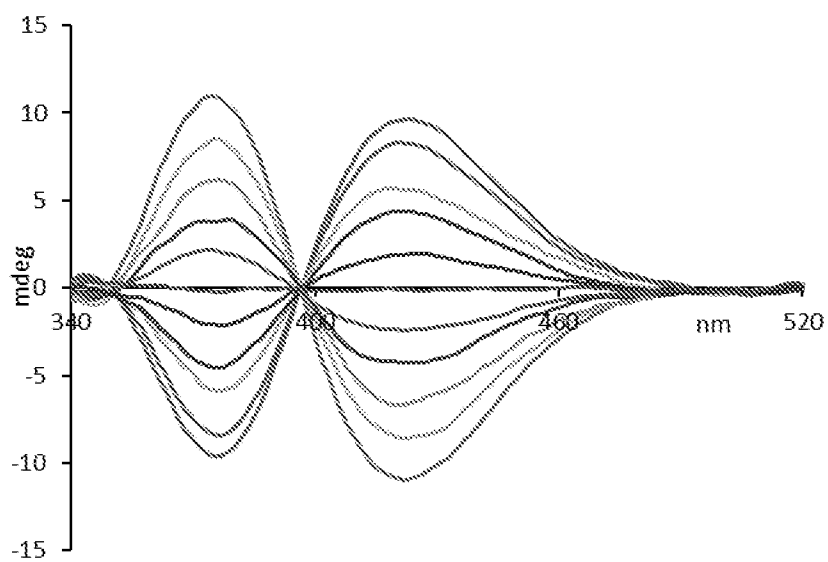


Figure 33A

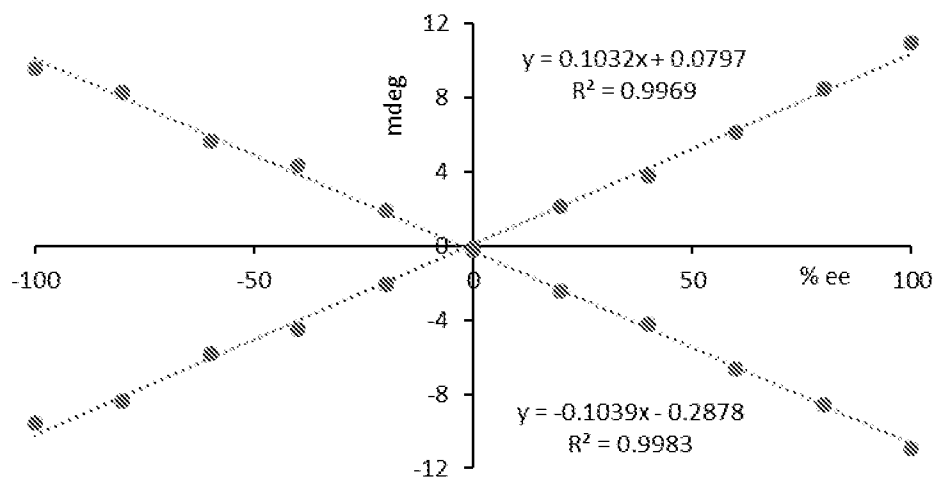


Figure 33B

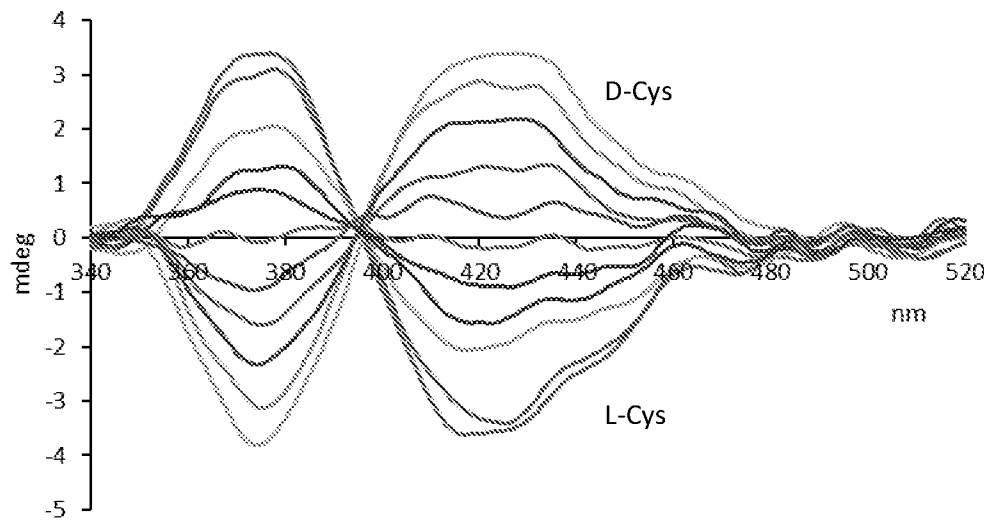


Figure 34

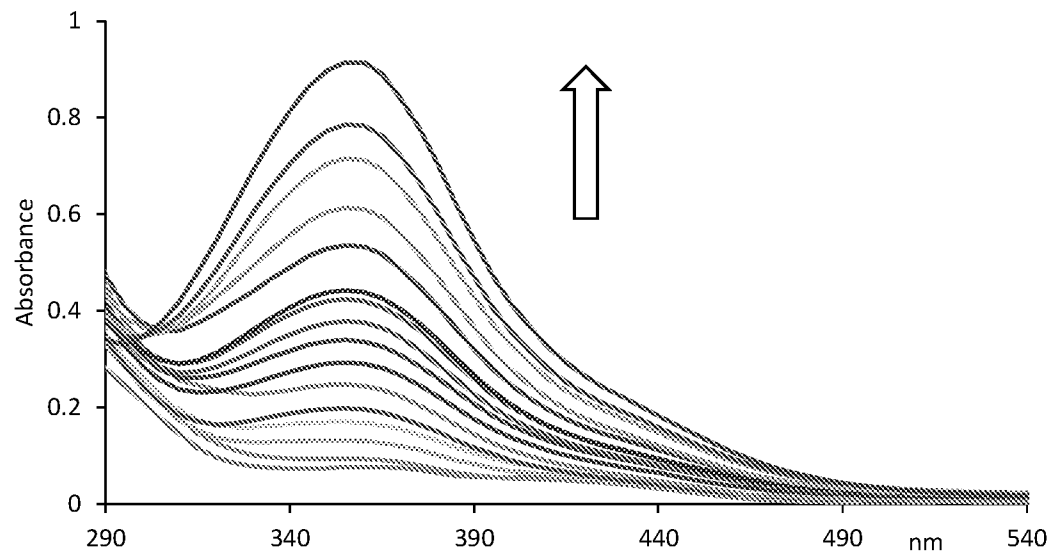


Figure 35A

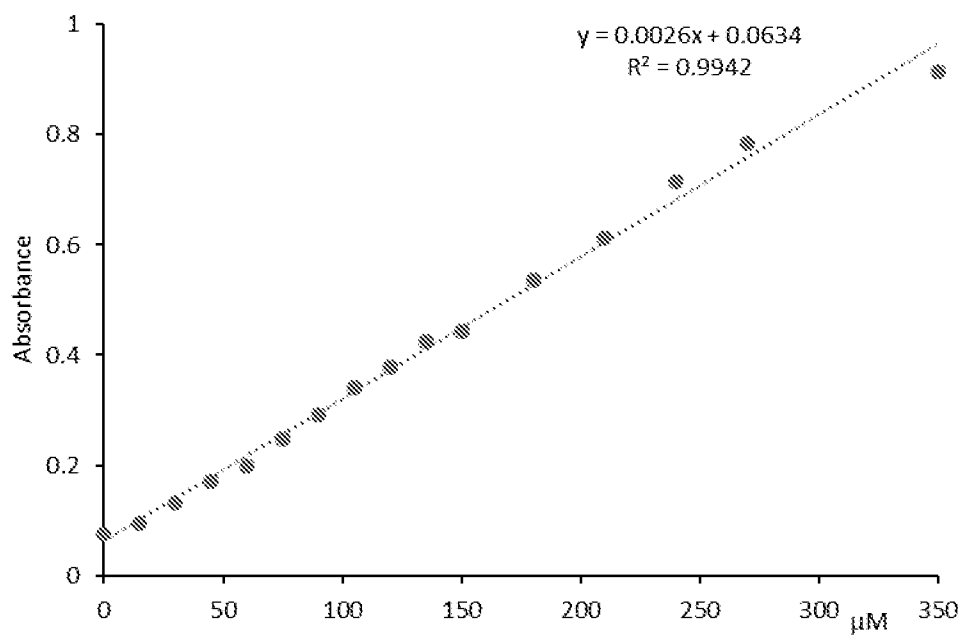


Figure 35B

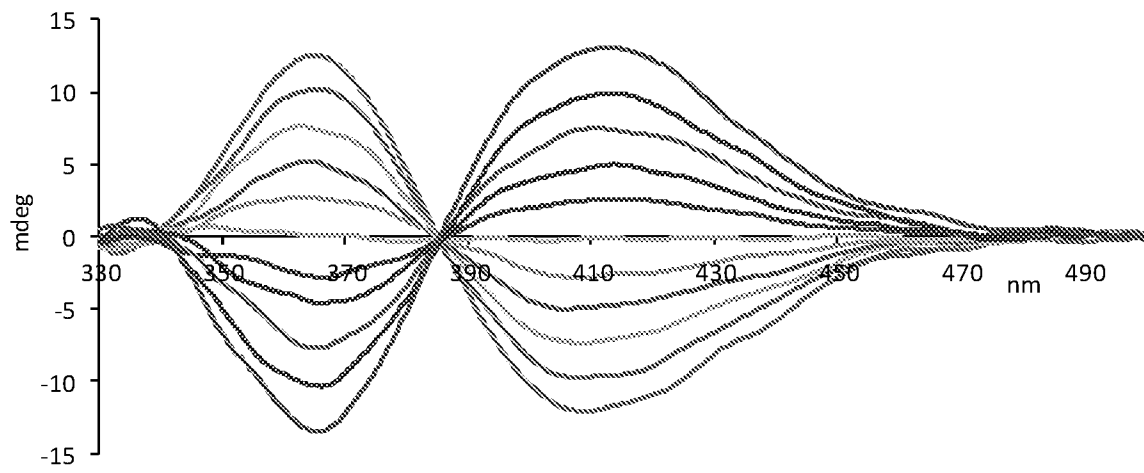


Figure 36A

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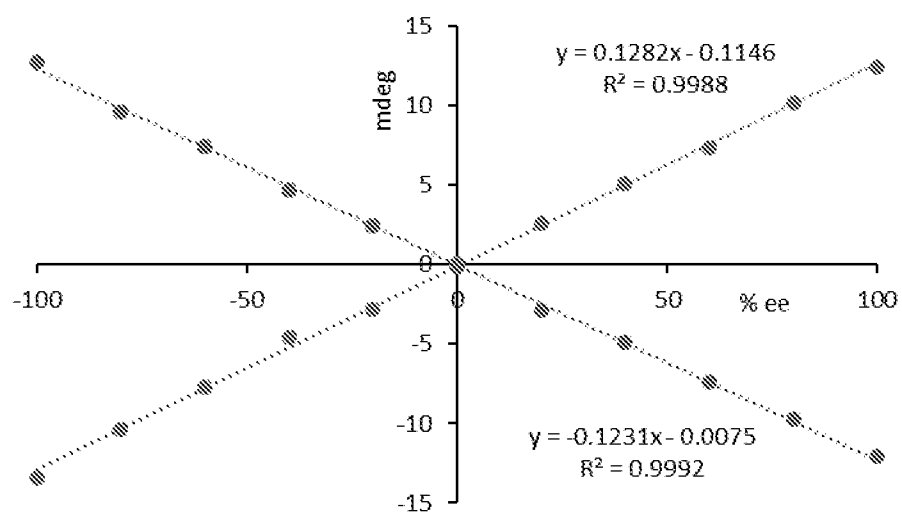


Figure 36B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/29109

A. CLASSIFICATION OF SUBJECT MATTER
 IPC - C07D 277/66; G01N 21/64, 33/52 (2018.01)
 CPC - C07D 277/66; G01N 21/64, 33/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PUBCHEM. CID 10008. 26 March 2005, pp. 1-32. Retrieved from the Internet <URL: https://pubchem.ncbi.nlm.nih.gov/compound/10008 >; page 3, formula	1-3, 6, 11
A	US 2015/0010938 A1 (OREGON STATE BOARD OF HIGHER EDUCATION ON BEHALF OF PORTLAND STATE UNIVERSITY) 08 January 2015; abstract	13-37
A	US 2016/0195532 A1 (THE SCRIPPS RESEARCH INSTITUTE) 07 July 2016; abstract; paragraph [0051]	13-37
A	CN 104610960 A (UNIV ZHEJIANG SCIENCE & TECH) 13 May 2015; English translation; abstract; claim 1	13-37
A	US 2016/0039792 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 11 February 2016; abstract	13-37



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 June 2018 (08.06.2018)

Date of mailing of the international search report

01 AUG 2018

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
 P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No. ..

PCT/US18/29109

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-Please Within the Next Supplemental Box-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 6, 11, 13-37

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

---Continued from Box No. III Observations where unity of invention is lacking---

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid. Groups I+, Claims 1-37 (in-part); compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is a leaving group selected from halogen; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H (first exemplary compound structure), and methods associated therewith.

The compound, compositions and methods will be searched to the extent the compound encompasses a compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is a leaving group selected from halogen; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H (first exemplary compound structure). Applicant is invited to elect additional compound(s), with fully specified structure (e.g. no optional or variable atoms or substituents) for each, to be searched. Additional compound(s) will be searched upon the payment of additional fees. It is believed that claims 1-3 (in-part), 6 (in-part), 11 (in-part), and 13-37 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass a compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is a leaving group selected from halogen; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H (first exemplary compound structure). Applicants must specify the claims that encompass any additionally elected compound structure(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is -CF₃; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H (first exemplary elected compound structure).

Groups I+ share the technical features including: a compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is a leaving group selected from halogen; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H.

However, these shared technical features are previously disclosed by the publication entitled "CID 10008" by PubChem (hereinafter 'PubChem').

PubChem discloses a compound comprising a chromophore of the formula, as shown, wherein: each X is C; each E is hydrogen; and LG is a leaving group selected from halogen; R1 and R2 are each -Ra wherein each Ra is H; and R3 is -Ra wherein Ra is H (compound as shown; page 3).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the PubChem reference, unity of invention is lacking.