Organic & Biomolecular Chemistry

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Journal:	*Organic & Biomolecular Chemistry*
Manuscript ID	OB-COM-01-2022-000198.R1
Article Type:	Communication
Date Submitted by the Author:	08-Mar-2022
Complete List of Authors:	Kariapper, Safia; Georgetown University, Department of Chemistry Thanzeel, F. Yushra; Georgetown University Zandi, Lily; Georgetown University, Department of Chemistry Wolf, Christian; Georgetown University, Department of Chemistry

Selective Chiroptical Sensing of D/L-Cysteine

F. Safia Kariapper, F. Yushra Thanzeel, Lily S. Zandi and Christian Wolf*

Department of Chemistry, Georgetown University, Washington, DC 20057, USA
cw27@georgetown.edu

Abstract: A chromophoric bifunctional probe design that allows selective chiroptical sensing of cysteine in aqueous solution is introduced. The common need for chiral HPLC separation is eliminated which expedites and simplifies the sample analysis while reducing solvent waste. Screening of the reaction between six phenacyl bromides and the enantiomers of cysteine showed that cyclization to an unsaturated thiomorpholine scaffold coincides with characteristic UV and CD effects, in particular when the reagent carries a proximate auxochromic nitro group. The UV changes and CD inductions were successfully used for determination of the absolute configuration, enantiomeric composition and total concentration of 18 test samples. This assay is highly selective for free cysteine while other amino acids, cysteine derived small peptides and biothiols do not interfere with the chiroptical signal generation.

The biological significance of amino acids is generally associated with their fundamental roles as building blocks of proteins and metabolic intermediates in biosynthetic pathways that generate hormones, neurotransmitters or other physiologically important compounds. Despite their structural simplicity, amino acids have many other pivotal functions in nature and affect human physiology and pathology in various ways that are still not fully understood. This is certainly the case with amino acids that appear in free form as nonracemic mixtures in mammalian tissue[1] and the central nervous system (CNS).[2] The variation in the concentration and enantiomeric ratio (*er*)

of amino acids in the CNS has been linked to schizophrenia, Parkinson's, Huntington's and Alzheimer's disease and other neurodegenerative or psychiatric disorders.[3] Not surprisingly, the apparent impact of D-amino acids on human health has attracted increasing attention within the life sciences[4] and sparked the development of sensing systems that can quantify D/L-amino acid mixtures, which has potential to assist with the diagnosis and study of several important diseases and ageing processes.[5]

To date, several chiral amino acids have been detected in both enantiomeric forms in mammalian cells. The natural appearance of the D-form and the biological significance of D/L-enantiomeric mixtures of cysteine are particularly noticeable.[6] The development of methods that achieve selective recognition and quantification of the enantiomers of cysteine in aqueous solution when other amino acids are present is undoubtedly challenging. While several assays that allow cysteine quantification have been reported, concomitant *er* determination is often not possible.[7] In our search for an optical probe that could overcome this limitation and allow quantification of the concentration and enantiomeric composition of cysteine in the presence of other amino acids including homocysteine, we found that phenacyl bromides have been used to synthesize unsaturated thiomorpholines[8] and other closely related pharmacophores with medicinal applications, Figure 1.[9] Inspired by these reports, we envisioned that phenacyl bromides, for example *o*-nitrophenacyl bromide, **1**, carrying an auxochromic *ortho*-nitro group which we have previously found to be beneficial for strong circular dichroism (CD) signal induction,[10] could be used as a cysteine specific probe. Under basic conditions, **1** was expected to undergo nucleophilic substitution by the thiol group followed by intramolecular imine condensation to form the heterocyclic product **2**. The 1,4-thiazine unit is formed via 6-*endo*-trig cyclization, which is favored by Baldwin's rules. We were delighted to discover that **1** gives a

distinct CD output upon smooth reaction with Cys exhibiting a strong maximum at approximately 330 nm, whereas the reaction mixtures with other amino acids, glutathione and homocysteine remain mostly CD silent**.** We now wish to report how this chemistry can be used for selective concentration and *er* determination of nonracemic cysteine mixtures.

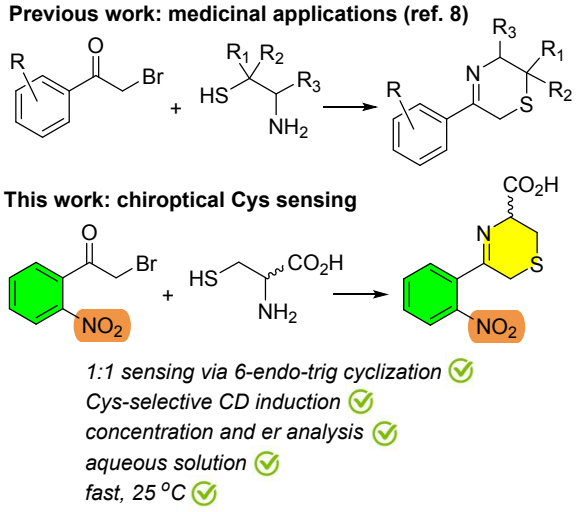

Figure 1. Development of a UV/CD sensing assay for D/L-cysteine mixtures.

To test our hypothesis, we initially screened the CD induction obtained by the reaction between enantiopure cysteine samples and the six commercially available phenacyl bromides **1-6** in aqueous solution in the presence of K_2CO_3, Na_2CO_3, NaOH, KOH, $NaHCO_3$ or Cs_2CO_3 as base or alternatively using potassium phosphate buffer at varying pH, see Figure 2 and ESI. The strongest CD inductions were measured with the probes displaying a nitro group in the *ortho* or *meta* position in aqueous acetonitrile containing two equivalents of either NaOH or KOH or when we employed a phosphate buffer at pH 8.5. The use of a mildly basic buffered solution seemed most attractive to us and we therefore chose these conditions and probe **1** for all further experiments. It is noteworthy that the reaction between cysteine and **1** gives a strong CD

amplitude at submillimolar concentrations with a maximum at approximately 330 nm. A strong CD signal above 300 nm is generally considered advantageous for quantitative sensing purposes and to avoid overlap with chiral impurities that typically have CD signatures at lower wavelengths. It has been reported that cysteine concentrations in healthy adults range from 15 to 30 μM while elevated levels seem to correlate with increased risk of Alzheimer's and heart disease.[11] The preparation of biological samples for this sensing assay might therefore require a routine lyophilization step prior to the analysis. HRMS analysis with cysteine and its methyl ester confirmed formation of the cyclization product shown in Figure 1 and CD monitoring showed that the reaction between **1** and cysteine was complete within 30 minutes, see ESI.

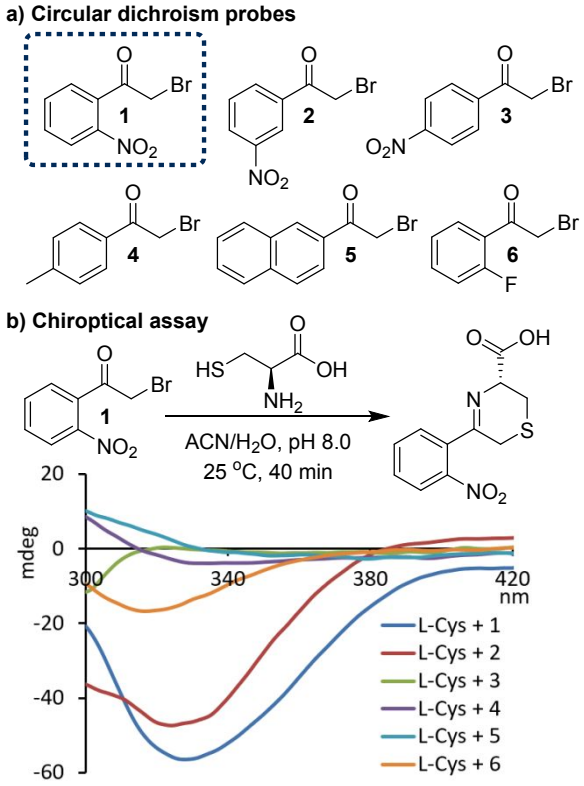

Figure 2. Structures of probes **1-6** and CD induction upon reaction with L-cysteine in acetonitrile/potassium phosphate buffer (pH 8.5) solution. All CD measurements were performed at 0.28 mM.

We then investigated the selectivity of our assay toward cysteine in the presence of other amino acids including homocysteine, Hcy, and the cysteine-derived tripeptide glutathione, Figure 3. Under the exact same conditions, the solutions containing **1** and the other amino acids or biothiols remained either CD-silent or showed very weak CD signals compared to the cysteine-derived unsaturated thiomorpholine scaffold. As expected, the sensing of the D- and L-Cys enantiomers gave opposite CD spectra with a maximum at 326 nm of the same intensity. Competition experiments in which the chiroptical probe, L-cysteine and an equimolar amount of either L-homocysteine, L-serine, L-alanine, L-tyrosine or glutathione were mixed and then subjected to CD analysis proved no interference from these potentially competing substrates on the chiroptical response. Interestingly, the assay is selective for free cysteine. We observed weak CD signals when L-cysteine methyl ester or L-CysGly were applied in the same sensing protocol, see ESI.

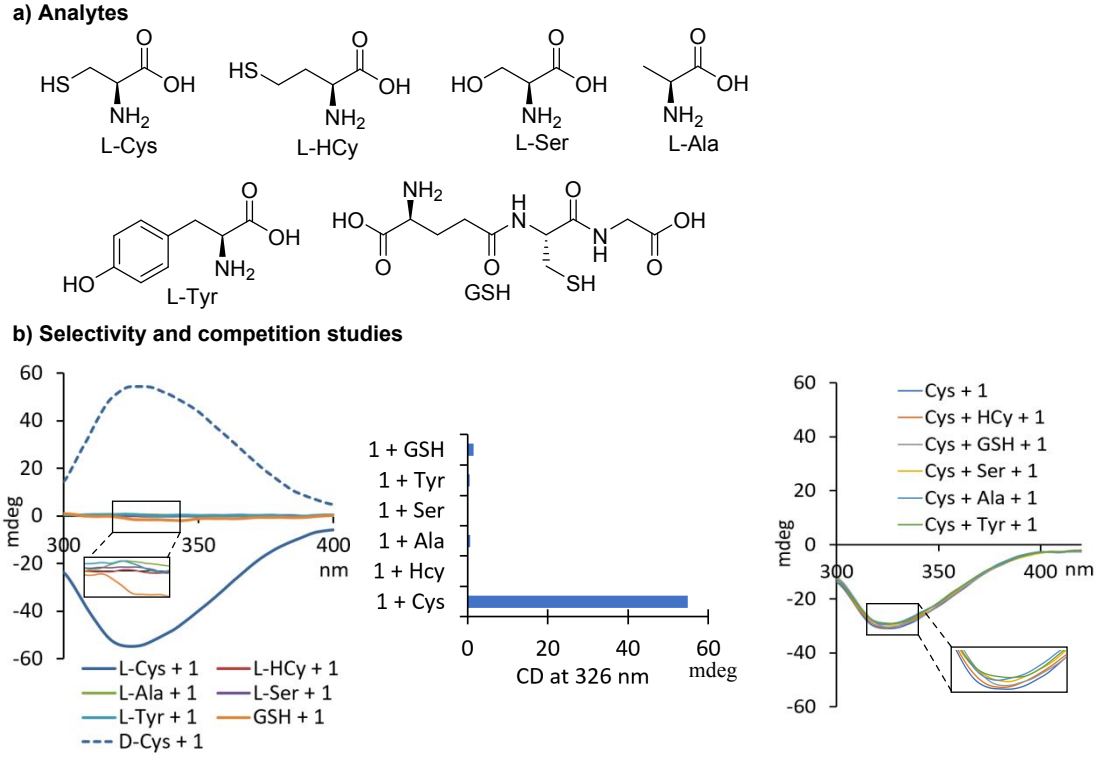

Figure 3. Structures of analytes tested (top) and determination of the assay selectivity for cysteine (bottom). The reactions with **1** were performed at 5.0 mM in acetonitrile/potassium phosphate buffer (pH 8.5) and all CD measurements were taken after dilution with acetonitrile to 0.24 mmol or at 0.14 mmol for the competition study.

An important feature of our probe is that it is achiral. The reaction with L- and D-cysteine therefore generates enantiomeric products but not diastereomers, contrary to chiral derivatizing agents (CDAs) which are often used for NMR spectroscopic enantiomer differentiation. The CD intensity induced by the reaction of cysteine with **1** can therefore be directly correlated to the enantiopurity of a sample while its sign reveals the absolute configuration of the predominant cysteine enantiomer. The use of an achiral probe capable of producing a strong CD response upon substrate binding such as **1** does not only simplify the analysis, it is also practical because it eliminates analytical problems that might arise from kinetic resolution effects or systematic inaccuracies that occur when a CDA is not 100% enantiopure.[12] Another essential probe design feature exploited in this study is that the reaction of **1** with Cys to the unsaturated thiomorpholine structure affords an altered chromophoric system. We suspected that this would result in a quantifiable UV change. Indeed, we observed a new red-shifted UV band at approximately 325 nm that proved to increase linearly with the cysteine concentration, Figure 4. Since this UV response must be independent of the substrate chirality (enantiomers have superimposable UV spectra and contribute equally to the measured absorption) we expected it to be applicable to concentration analysis irrespective of the enantiomeric sample composition. Similarly, we observed that the magnitude of the generated CD signals increase linearly with the sample enantiomeric excess (*ee*).

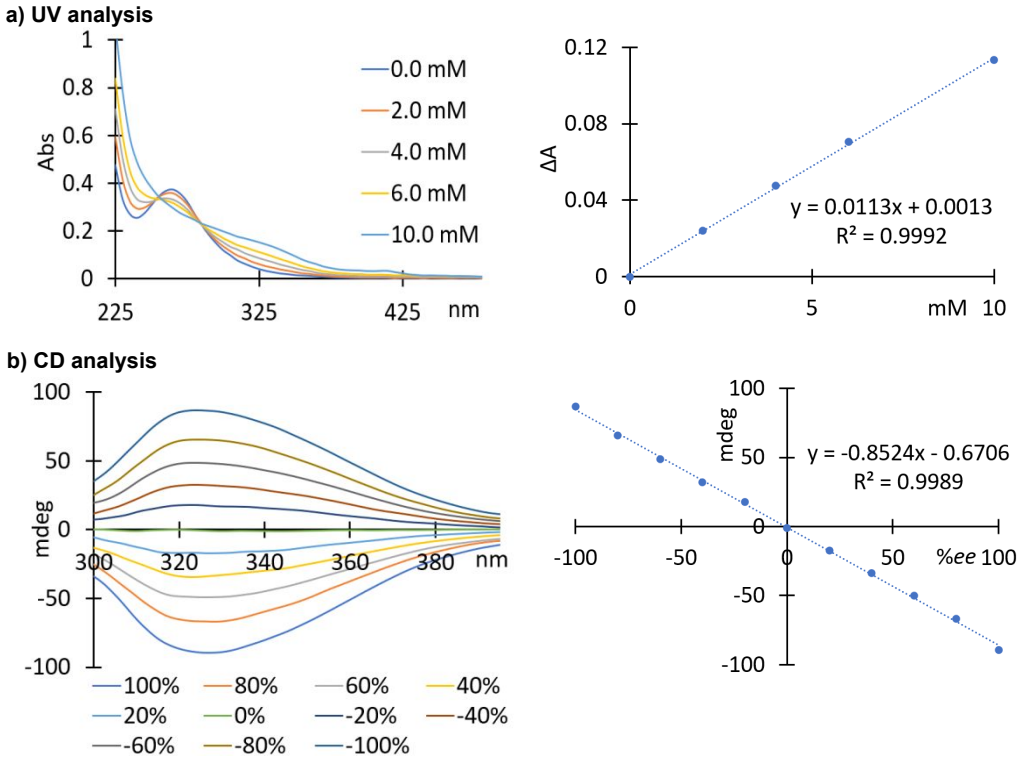

Figure 4. Quantitative UV and CD analysis. a) A solution of **1** (10.0 mM) and cysteine in varying concentrations (0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mM) was stirred for 40 minutes. The UV data were collected after dilution of 30.0 µL of the mixtures in 6.0 mL of acetonitrile (left). The difference in the UV intensity of the reaction product and free **1** at 325 nm is plotted versus the cysteine concentration (right). b) The change in the CD response of **1** to cysteine mixtures of varying enantiomeric composition was determined. CD measurements were obtained at 0.57 mM (left) and a linear relationship between the CD amplitudes at 326 nm versus sample %*ee* was obtained (right). Negative %*ee* values where assigned to solutions containing D-cysteine as the major enantiomer.

Finally, we wanted to test the usefulness of our chiroptical assay for determination of the absolute configuration, enantiomeric ratio and total concentration of arbitrarily prepared cysteine

samples. The collection of an accurate UV spectrum is significantly faster than a full-range CD measurement, and we therefore decided to use only a small window from 320 to 330 nm for the latter. To just capture the region including the induced CD maximum is sufficient for the quantitative application and it saves considerable time. A total of 18 samples were analyzed with **1** as described above and the UV and CD data were then quantified using the previously obtained calibration curves. All samples were analyzed in duplicate. The spectra of five representative samples (#5, 9, 10, 14 and 15) are exemplarily shown in Figure 5. We found that the corresponding UV and CD curves obtained for each sample are perfectly superimposable which demonstrates the high precision of this assay.

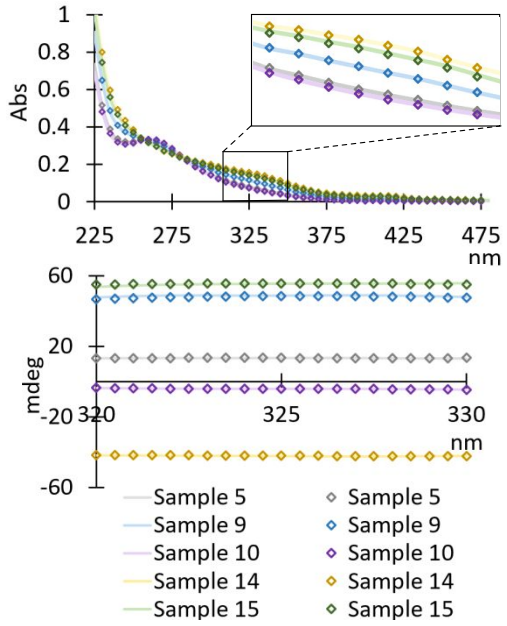

Figure 5. UV curves (top) and CD excerpts (bottom) of selected samples treated with **1**. See Table 1 and ESI for sensing results and details.

We were very pleased with the high accuracy of the chiroptical sensing results, Table 1. In all cases, the correct absolute configuration of the major cysteine enantiomer was assigned based

on the sign of the induced CD signal. The enantiomeric ratios and sample concentrations were determined with relatively small error margins. For example, sensing of sample #1 containing D/L-Cys in 40:60 *er* at 9.0 mM gave the exact same values and the composition of sample #2 (82.0:18.0 *er*, 6.0 mM) was determined as 78.8:21.2 *er* and 5.9 mM. Comparison of the results obtained with samples #3, 9, 10 and 18 show that the assay performs equally well with mixtures having low or very high enantiomeric ratios.

Table 1. Chiroptical sensing of the absolute configuration, enantiomeric ratio and concentration of 18 cysteine samples.

	Sample composition			**Sensing results**		
Sample #	**Configuration (major enantiomer)**	***Enantiomeric ratio***	**Concentration (mM)**	**Configuration (major enantiomer)**	***Enantiomeric ratio***	**Concentration (mM)**
1	L	40.0:60.0	9.0	L	40.0:60.0	9.0
2	D	82.0:18.0	6.0	D	78.8:21.2	5.9
3	L	2.0:98.0	5.0	L	3.9:96.1	4.8
4	L	35.0:65.0	5.5	L	34.3:65.7	5.4
5	D	70.0:30.0	4.0	D	72.8:27.2	3.5
6	D	80.0:20.0	4.5	D	82.1:17.9	4.2
7	L	16.0:84.0	8.0	L	18.3:81.7	7.9
8	D	73.0:27.0	6.5	D	71.1:28.9	6.4
9	D	92.0:8.0	7.0	D	93.6:6.4	6.6
10	L	44.0:56.0	3.5	L	41.8:58.2	2.8
11	L	39.0:61.0	8.5	L	39.3:60.7	8.4
12	D	72.0:28.0	9.5	D	68.7:31.3	9.3
13	L	41.0:59.0	5.0	L	39.7:60.3	4.8
14	L	24.0:76.0	10.0	L	26.3:73.7	10.3
15	D	90.0:10.0	9.0	D	85.9:14.1	9.2
16	D	60.0:40.0	4.5	D	59.5:40.5	4.4
17	L	16.0:84.0	5.0	L	13.7:86.3	5.1
18	L	44.0:56.0	7.5	L	44.1:55.9	7.3

See ESI for details.

In conclusion, we have introduced a highly cysteine-selective chiroptical sensing assay that uses readily available *o*-nitrophenacyl bromide as UV/CD probe in aqueous solutions. The reaction with free cysteine gives characteristic UV changes and a strong CD induction above 300 nm which can be correlated to the sample concentration and enantiomeric composition. Competition studies proved that other amino acids and biothiols do not interfere with this assay and the usefulness for quantitative cysteine analysis was successfully verified with 18 samples. This approach eliminates the common need for chiral HPLC separation, which accelerates and simplifies the sample analysis while reducing solvent waste. This optical sensing method can easily be adapted by academic and industrial laboratories and is amenable to automated high-throughput microplate readers if many samples need to be analyzed in parallel.

Conflicts of interest. There are no conflicts to declare.

Acknowledgement. We gratefully acknowledge financial support from the U.S. National Science Foundation (CHE-1764135).

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