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PII: S0026-265X(18)30476-4
DOI: doi:10.1016/j.microc.2018.05.048
Reference: MICROC 3197

To appear in: *Microchemical Journal*

Received date: 7 May 2018
Revised date: 25 May 2018
Accepted date: 25 May 2018

Please cite this article as: Salvatore Sotgia, Angelo Zinellu, Mauro Forteschi, Panagiotis Paliogiannis, Giovanni A. Deiana, Gerard A. Pinna, Arduino A. Mangoni, Ciriaco Carru , A liquid chromatography-mass spectrometry study on the spirocyclization of ninhydrin with the aminothiols. Microchemical Journal(2017), doi:10.1016/j.microc.2018.05.048

# A liquid chromatography-mass spectrometry study on the spirocyclization of ninhydrin with the aminothiols

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**Abstract**

Ninhydrin reacts with some aminothiols to form spiranes adducts whose optical and chromatographic properties have proved to be useful for chiral recognition. Liquid chromatography-mass spectrometry data along with spectroscopic analysis reveal that under certain conditions, in addition to the known single-spirane configuration, the spirothiazolidinic complexes can exist also as double- and mixed double-spiranes. The reaction was exploited to check the enantiomeric purity of two commercially available dosage form of D-penicillamine and to measure the aminothiol concentration in the urine sample from a subject under treatment with the drug. Separation of diastereoisomers was achieved on a C18 column in isocratic mode by using a mixture of an aqueous solution of formic acid (30 mmol/L)/acetonitrile (90:10, v/v) as a mobile phase. Diastereoisomers were detected by a fluorescence detector and mass spectrometer in short times and with a good resolution. Intra- and inter-assay reproducibility were under 4% with an average recovery of 98%. At a LOD of 0.01%, no evidence of the toxic distomer (L-enantiomer) was found in the biological sample and drugs.

Keywords: Spiranes; Spirothiazolidine; Chiral; Diastereoisomers.

**Introduction**

Ninhydrin reacts with some aminothiols differently than it does with other amino acids. With the latter, the reaction normally leads to the production of the well-known purple-colored compound Ruhemann's purple [1], whose optical properties have been widely used for qualitative and quantitative analysis of amino and imino acids [2]. By contrast, with aminothiols such as penicillamine, cysteine, or cysteamine, the reaction does not proceed to completion or it occurs only under certain condition [3]. Kuhn and Hammer firstly explained this unusual behavior as a consequence of the formation of a colorless condensation product instead of the chromogenic Ruhemann's purple [~~3~~ 4]. This was confirmed by Friedman and Sigel, who identified the alternative compound as a thiazine derivative [~~4~~ 5]. However, Prota and Ponsiglione challenged this assertion, demonstrating that the low reaction rate between cysteine and ninhydrin, signaled by the low color yield, was due to a competitive nucleophilic displacement of the amino and sulfhydryl groups of the aminothiol on the ninhydrin molecule, forming a spirane derivative rather than a thiazine (figure 1) [~~5~~ 6]. Similar reactions occurred with cysteine analogs such as penicillamine and cysteamine. Based on this reaction, we previously developed a derivatization method coupled to chiral ligand-exchange high-performance liquid chromatography (CLEC) for the detection of the spirothiazolidinic derivatives of penicillamine isomers by means of optical detectors [~~6~~ 7]. The spirane adducts, in fact, have optical properties that allow the detection of derivatized isomers with high sensitivity using either UV-Vis or fluorescence detectors. While attempting to improve such method, we observed, under certain conditions, a further unusual reaction between ninhydrin and aminothiols that makes ninhydrin suitable as a chiral derivatizing agent. As a result, we developed an original method for the indirect reversed phase HPLC recognition of stereoisomers of penicillamine and other aminothiols such as cysteine and cysteinylglycine [~~7~~ 8]. The derivatization of disulfides and mixed disulfides would easily explain this undocumented feature of ninhydrin. However, based on experimental evidence, we excluded in our previous work that the reaction

involves the formation of disulfides and mixed disulfides between the aminothiols. Considering the ability of ninhydrin to spirocyclize with the aminothiols, therefore, we hypothesized that the diastereoisomers could be formed by double- and mixed double-spiranes. Thus, this study aims to obtain data to support or reject such configuration. The analytical potential of the derivatization reaction was evaluated analyzing the penicillamine content in urine sample from Wilson's disease patient administered for more than 30 years with 1.2 g daily of D-Penicillamine. Moreover, the enantiomeric purity of two commercially available dosage form of D-penicillamine was also assessed. An ultra-performance liquid chromatograph coupled to UV-Vis and fluorimeter detectors, as well as to electrospray ionization-tandem mass spectrometer was employed for these purposes.

## Experimental

### *Chemicals and reagents*

Acetonitrile (ACN) and ethanol (EtOH), both HPLC grade, formic acid, pure enantiomers (D-Pen, L-Pen) and racemate (DL-Pen) of penicillamine, L-Cysteine (L-Cys), and ninhydrin were purchased from Sigma Aldrich Italia (Milan, Italy). Pharmaceutical formulations of D-Pen were from Ely Lilli Italia (Milan) and Stabilimento Chimico Farmaceutico Militare (Florence, Italy). High-purity water, obtained from a Millipore Milli-Q system (Merck Millipore, Milan, Italy), was used throughout the experiments.

### *Solutions*

A weighed amount of ninhydrin was dissolved in EtOH to get a 2% (w/v) solution. DL-Pen, L-Pen, and D-Pen were prepared as stock solutions, at concentrations of 20 mmol/L, in ultrapure water, and stored at -80 °C until use. For the analysis of pharmaceutical formulation, a 150 mg D-penicillamine capsule containing the excipients lactose and magnesium stearate, was dispersed in ultrapure water to give a sample stock solution of approximately 500 mmol/L which was stored at −80 °C until use. Fresh working solutions of aminothiols were prepared by diluting the stock

solutions in ultrapure water. L-Cys was freshly prepared in ultrapure water at a concentration of 160 mmol/L.

***Participants to study and samples collection***

After informed written consent was obtained, urine samples from one volunteer with no medical history and from Wilson's disease patient under treatment with D-Pen were collected at the same time of the day. A 1.5-mL volume was centrifuged at 17.000 x g for 10 min at room temperature then an aliquot~~s~~ derivatized following procedure 2.

***Apparatus and chromatographic conditions***

Chromatographic experiments were performed on a Waters Acquity UPLC system equipped with Waters Acquity UPLC fluorescence (FLD) and Photodiode Array eλ (PDA) detectors, as well as a Waters Acquity UPLC tandem quadrupole mass spectrometer (TQD) (Waters Italia, Milan, Italy). The separation was achieved on a 2.5 µm 100 × 2.1 mm Waters XBridge BEH XP C18 column that received isocratically a mobile phase composed of a mixture of an aqueous solution of 30 mmol/L formic acid/ACN (90:10, v/v) at a flow rate of 0.5 mL $min^{-1}$~~)~~. The column compartment was maintained at 40 °C and the samples were held at 23 °C in the autosampler. Injection volume was 2 µL and effluent was monitored both by PDA (from 200 to 500 nm) and FLD (excitation wavelength 391 nm, emission wavelength 415 nm) detectors as well as by mass spectrometer. The latter was used in full-scan mode in the ESI-positive ionization mode. The desolvatation and source temperatures were, respectively, 500 and 150 °C. The capillary and cone voltages were set at 3.5 kV and 31 V, respectively. Nitrogen, used as desolvation and cone gas, was delivered, respectively, at 30 L $h^{-1}$ and 600 L $h^{-1}$.

***Derivatization procedure***

Derivatization was performed according to the procedures previously described by our group, with minor modifications [~~6,7~~ 7,8]. In procedure 1, a 200 µL volume of an aqueous racemic or D- or L-

Pen standard solution was mixed with 20 µL of the ethanolic 2% (w/v) ninhydrin solution. After vortex-mixing, the reaction mixture was left for 5 min at 100 °C in a thermoblock heater. In procedure 2, a 200 µL volume of an aqueous 160 mmol/L L-Cys solution was mixed with 100 µL of standard solutions of racemic (DL-Pen) or enantiomeric (D-Pen, L-Pen) penicillamine. After vortex-mixing, 20 µL of the ethanolic 2% (w/v) ninhydrin solution were added, then the reaction mixture was left for 5 min at 100 °C in a thermoblock heater.

**Results and discussion**

Prota and Ponsiglione explained the exception of cysteine and cysteine-like compounds to the general reaction of α-amino acids with ninhydrin as due to the interruption of the reaction when the intermediate formed by the interaction of the ninhydrin with the α-amino function, further interacting with the β-thiol group, is trapped into a spirane complex (figure 1) [~~5~~ 6]. As showed in figure 2, the HPLC of a standard solution of DL-Pen following derivatization by procedure 1, shows that three products are obtained. The observed mass-to-charge ratio for P3 of *m/z* 292.34 is consistent with the single-spirane form originally proposed by Prota and Ponsiglione as the product formed by the reaction between ninhydrin and penicillamine. Because the spirane keeps the original chirality of the aminothiol, P3 can exist as D- or L-enantiomer depending on the penicillamine isomer trapped into the adduct. Interestingly, the mass data for P1 and P2 of *m/z* 423.41 suggest a possible double, in addition to the known single, spirane configuration. This can be explained assuming the reactivity of the carbons in position 1 or 3 of the ninhydrin, in addition to the carbon in position 2. The chromatographic behavior of P1 suggests that it consists of a molecule of ninhydrin coupled to two molecules of D- or L-Penicillamine. P2, instead, would consist of a molecule of ninhydrin linked to two molecules of a mix of D- and L-Penicillamine. As expected for a pair of enantiomers, in fact, the retention time for P1 should be the same regardless of the single enantiomer incorporated in the adduct. P2, being a diastereoisomer compared to P1, in addition to having a different retention time, should only be formed when both enantiomers are present in the

reaction mixture. Indeed, as displayed in figure 3, the retention time at 7.93 min for P1 is the same either using L-Pen or D-Pen. By contrast, when the D-Pen and L-Pen were derivatized individually, P2 was missing in all the chromatographic tracks (figure 3). Compared to P1 and P2, P3 shows different optical properties. The former showed an absorption spectrum with maximum wavelengths at 206, 225, 258, and 391 nm (figure 4) and fluorescence features mainly detectable on excitation at 391 nm and emission at 415 nm. Conversely, P3 showed no fluorescence upon excitation at different wavelengths and a maximum wavelength of 230 nm along with a shoulder around 260 nm (figure 4). The lack of fluorescence for P3, which refers to the single-spirane form, suggests that the double-spirane configuration is needed for the fluorescence. Therefore, we now speculate that in our previous work based on CLEC, we separated the double-, rather than the single-, spirane forms of the adducts [~~6~~ 7]

Based on this experimental evidence, we speculated that the ability of the aminothiols and ninhydrin to form double-spiranes might provide a novel opportunity for chiral recognition as the double-spiranes may also be formed as a mix of enantiomers between different aminothiols. In other words, an additional chiral aminothiol, e.g., L- or D-cysteine, added to a mixture of penicillamine enantiomers, would act as a chiral selector thus making ninhydrin suitable as a chiral derivatizing reagent. Indeed, we have already exploited this unexpected feature previously [~~7~~ 8]. A derivatization method (procedure 2) was, in fact, developed for the indirect chiral recognition of stereoisomers of penicillamine and other aminothiols such as cysteine and cysteinylglycine. Although the structural details were unknown at that time, experimental data suggested that compounds looking like diastereoisomers were not made up of disulfides and/or mixed disulfides. This hypothesis has been confirmed in this study. As showed in figure 5, the derivatization of a racemic mixture of penicillamine by procedure 2 leads to the formation of five adducts with mass data that are consistent with the mixed- and double-spiranes rather than to the disulfides. In addition, all the obtained compounds show fluorescence and absorption spectrum superimposable to that of the P1 and P2 obtained with procedure 1 thus giving further support to the double-spiranes

configuration. Mass-to-charge ratios for the five adducts were *m/z* 367.21 for P1b, and *m/z* 395.25 for the remaining products P2b, P3b, P4b, and P5b. Mass-to-charge ratio of P1b was consistent with the enantiomeric double-spirane form consisting of ninhydrin and two molecules of L-Cys (figure 6). As L-Cys is a reagent in procedure 2, P1b was always detectable in all chromatograms (figure 5, a, b, and c). Unexpectedly, the single-spirane form, consisting of ninhydrin and one molecules of L-Cys, was undetectable both by UV-Vis detector and mass spectrometer. The reason could be related to the consumption of cysteine to form both the double-spirane (Cys/Cys) and the mixed double-spirane with penicillamine (Cys/Pen). P2b and P3b were both detectable in the chromatogram related to the racemic mixture of penicillamine (figure 5, a). As expected, P2b was also detectable in the chromatogram referring to L-Pen (figure 5, b) while P3b to that referring to D-Pen (figure 5, c). Mass-to-charge ratios for such adducts were consistent with the mixed double-spiranes consisting of ninhydrin, a molecule of L-Cys and one of D- or L-Pen (figure 6). In particular, P2b contains the L-Pen enantiomer while P3b the D- one. Differently from our previous work, P4b and P5b were chromatographically unresolved. This could be explained by the use of formic acid instead of propionic acid in the mobile phase. As the latter did not allow sufficient ionization of the adducts in the ESI spray, in this work it was satisfactorily replaced by formic acid. Regardless of the chromatographic shortcomings, P4b and P5b showed the same detectability of P2b and P3b, as well as the same mass-to-charge ratios. Curiously, retention times were reversed compared to the first ones with the L-enantiomer (P4b) slower than D- (P5b). Structural isomerism likely may account for the presence of these additional pairs of diastereoisomers.

The analytical potential of the reaction was investigated in this work analyzing urine samples from a subject under treatment with D-Pen and an apparently healthy volunteer. Quantification was performed exploiting the fluorescence features of the first pair of adducts (P2b and P3b). The method was linear in the range from 70 to 1120 µmol $L^{-1}$ ($R>0.999$) both for D- and L-Pen. Intra- and inter-assay reproducibility expressed as relative standard deviation (RSD%) of triplicates of a racemate solution of penicillamine were under 4% with an average recovery of 98%. LOD and

LOQ were computed on two calibration curves using the following equations 3.3σ/S and 10σ/S, respectively, where σ is the standard deviation of the intercept and S the slope of the calibration plot. The average LOD and LOQ were 20.52 and 62.18 μmol $L^{-1}$, respectively. As expected and displayed in figure 7, no evidence of D- or L-Pen was found in the urine sample from the healthy subject and in a blank solution. On the contrary, D-Pen concentration in the sample from Wilson's disease patient was determined to be 290.20±15 μmol $L^{-1}$. No trace of L-Pen was detected in the biological sample, thus excluding both a chiral inversion in vivo or the occurrence of distomer in the drug. In our previous work by the procedure 2 and FLD, the lowest detectable amount of penicillamine distomer in samples of the D-enantiomer, evaluated by a signal-to-noise ratio of 3:1, was 0.01%. At this LOD level, the enantiomeric purity of two penicillamine formulations was confirmed in this work, as it was possible to detect only the non-toxic and pharmacologically safe D- eutomer.

## 4. Conclusion

In summary, we perform a mass spectrometry characterization of the adducts formed during an unusual reaction between ninhydrin and aminothiols discovered earlier. Mass data reveal that in addition to the known single configuration, double- and mixed double-spiranes are also possible. This feature making ninhydrin suitable as a chiral derivatizing agent. As far as we know, a such reaction mechanism was hitherto unknown. The reaction was found to be useful for the quality control of bulk material and formulations as well as for the measure of penicillamine in biological specimens.

**Acknowledgements**

Professor Arduino A. Mangoni contributed to this study during a Visiting Professorship at the University of Sassari.

**Compliance with ethical standards**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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**Legends**

**Figure 1.** Thiazinic and spirothiazolidinic structures as proposed, respectively, by Friedman and Sigel and Prota and Ponsiglione.

**Figure 2.** Elution profile of a racemic mixture of penicillamine derivatized by procedure 1 and detected by a) UV-Vis detector and b) fluorimeter.

**Figure 3.** Elution profiles of L-Pen (track a) and D-Pen (track b) solutions derivatized by procedure 1 and detected by a) UV-Vis detector and b) fluorimeter.

**Figure 4.** Absorbance and emission spectra ~~spectrum~~ of the a) adducts P1, P2, P1b, P2b, P3b, P4b, and P5b and absorbance spectrum b) of the adduct P3.

**Figure 5.** Elution profiles of DL-Pen (track a), L-Pen (track b), and D-Pen (track c) solutions derivatized by procedure 2 and detected by fluorimeter.

**Figure 6.** Proposed structures for the single-, double-, and mixed double-spiranes.

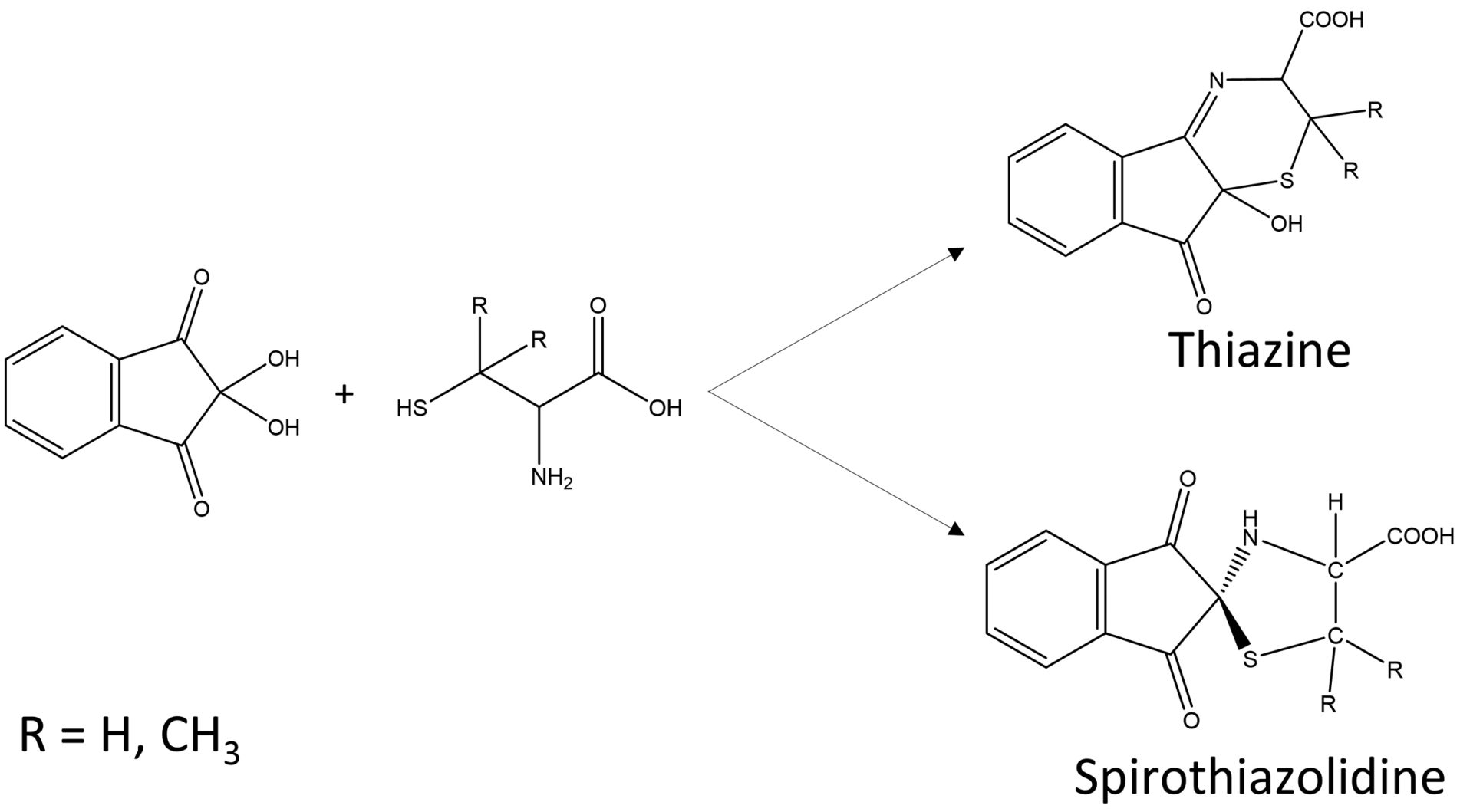
**Figure 7.** Representative chromatograms of a) a biological sample from a Wilson's disease patient, b) a blank, and c) of a urine sample from a volunteer.

## Highlights

Double- and mixed double-spiranes between ninhydrin and aminothiols were characterized;

Double- and mixed double-spiranes have fluorescent features;

Double- and mixed double-spiranes configurations make ninhydrin suitable as a chiral derivatizing reagent;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OH
OH
O
+
R
R
O
HS
OH
NH2
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N
R
R
S
OH
O
Thiazine
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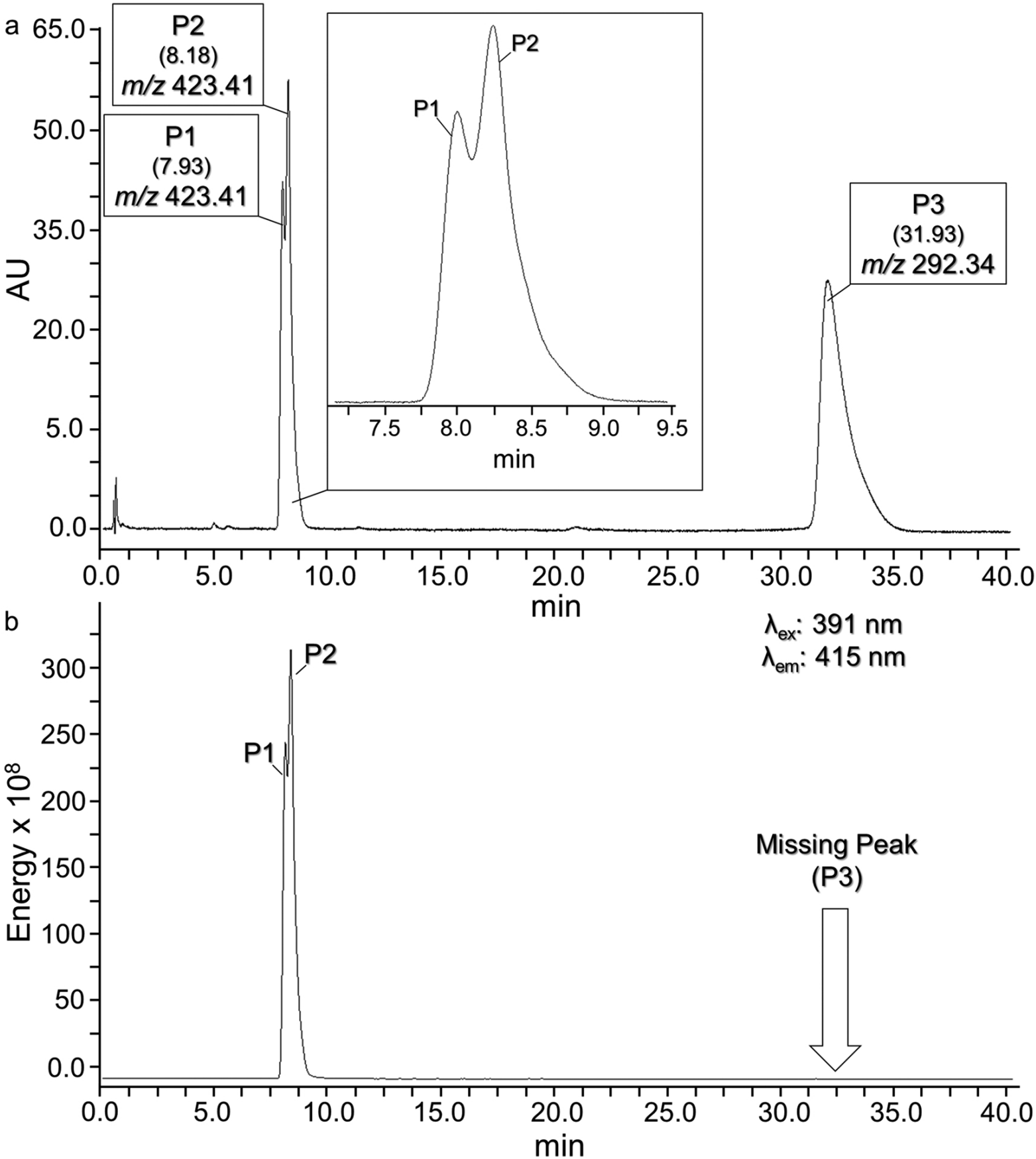
H
HN
COOH
C
S
C
R
R
O
Spirothiazolidine
R = H, CH3

Figure 1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P1
(7.93)
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P3
(31.93)
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min
b
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λem: 415 nm
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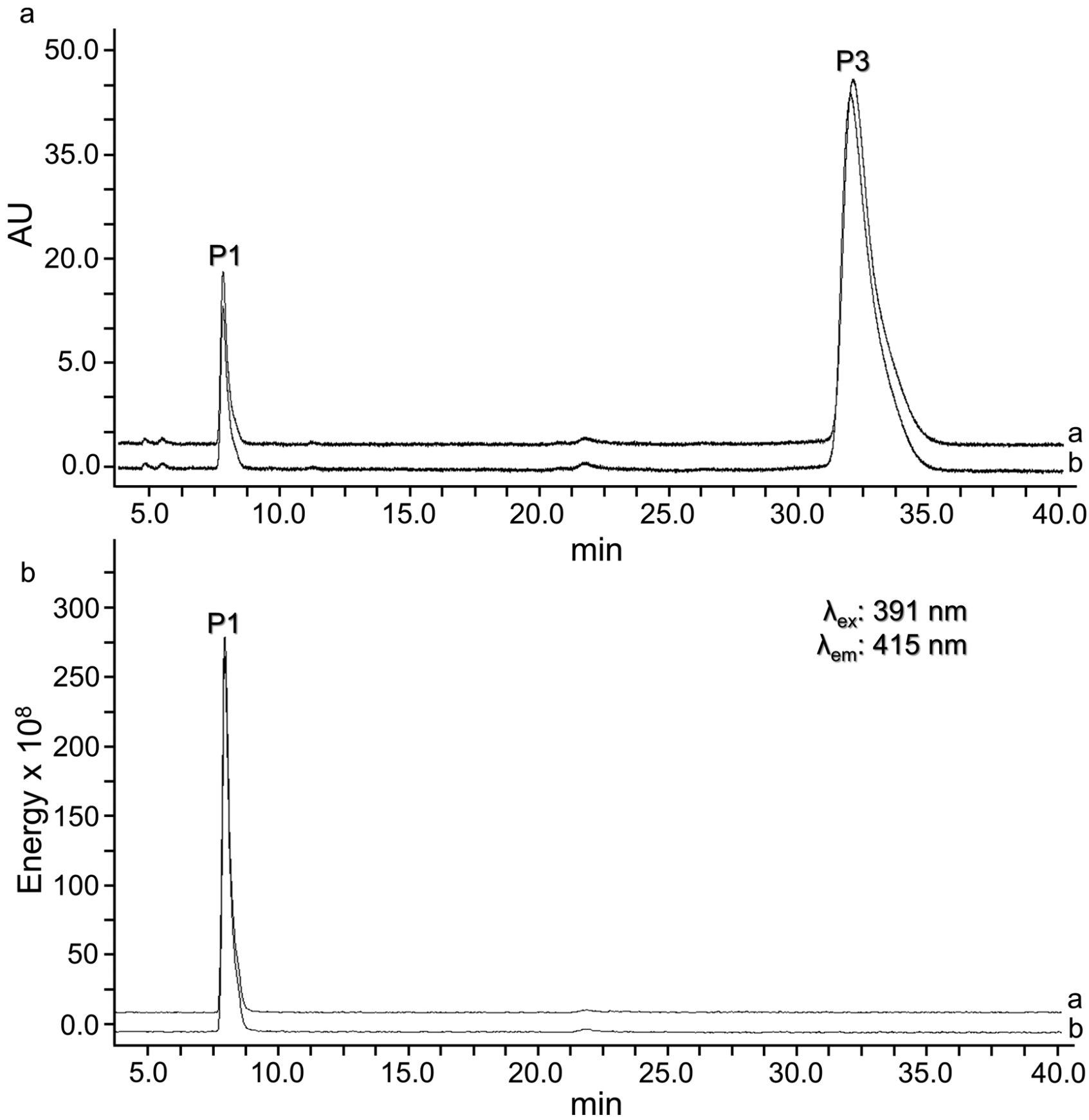
(P3)
min

Figure 2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P3
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20.0
25.0
30.0
35.0
40.0
min
b
300
250
200
150
100
50
0.0
Energy x 10^8
P1
λex: 391 nm
λem: 415 nm
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b
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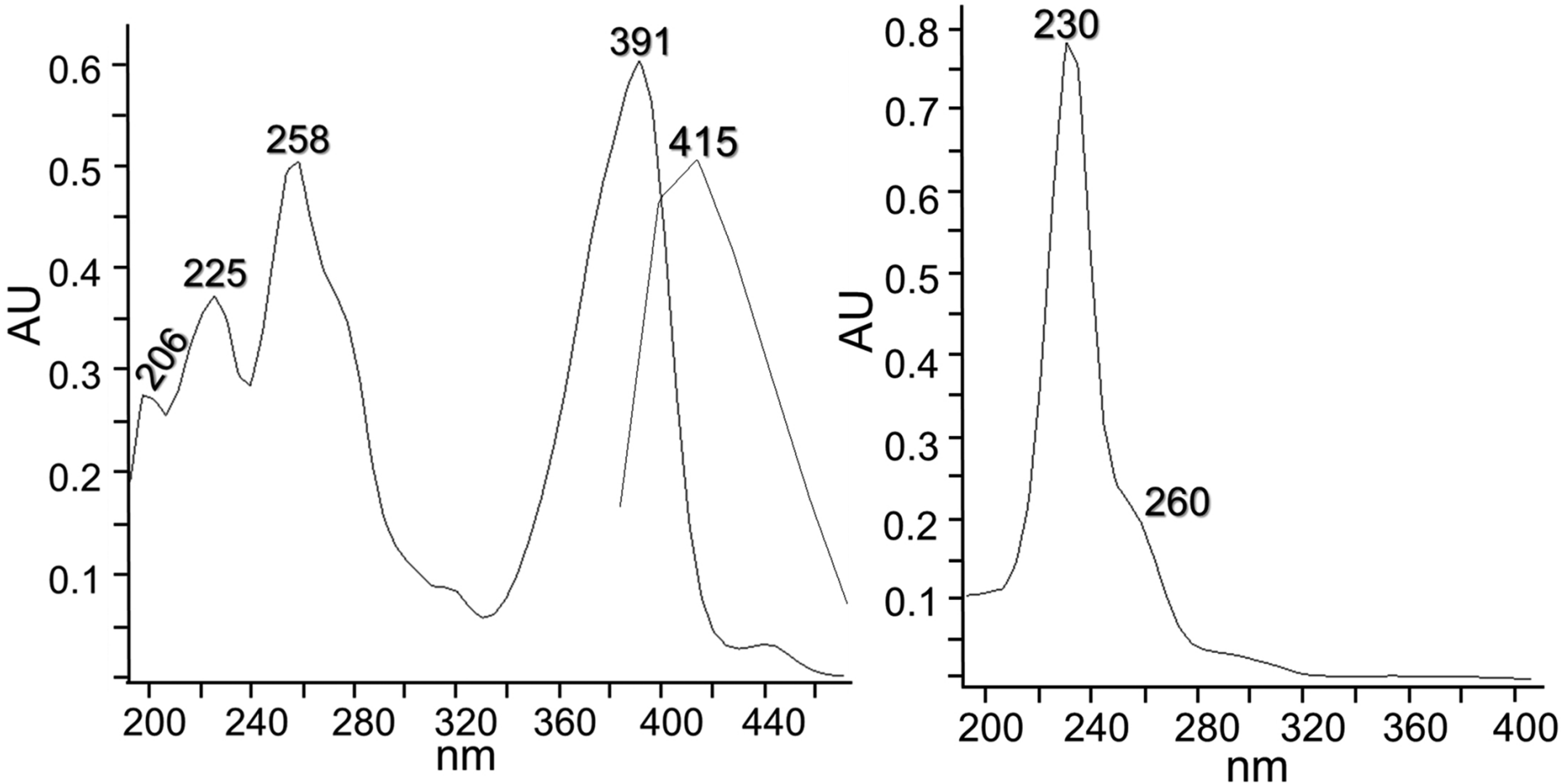
40.0
min

Figure 3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nm
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0.7
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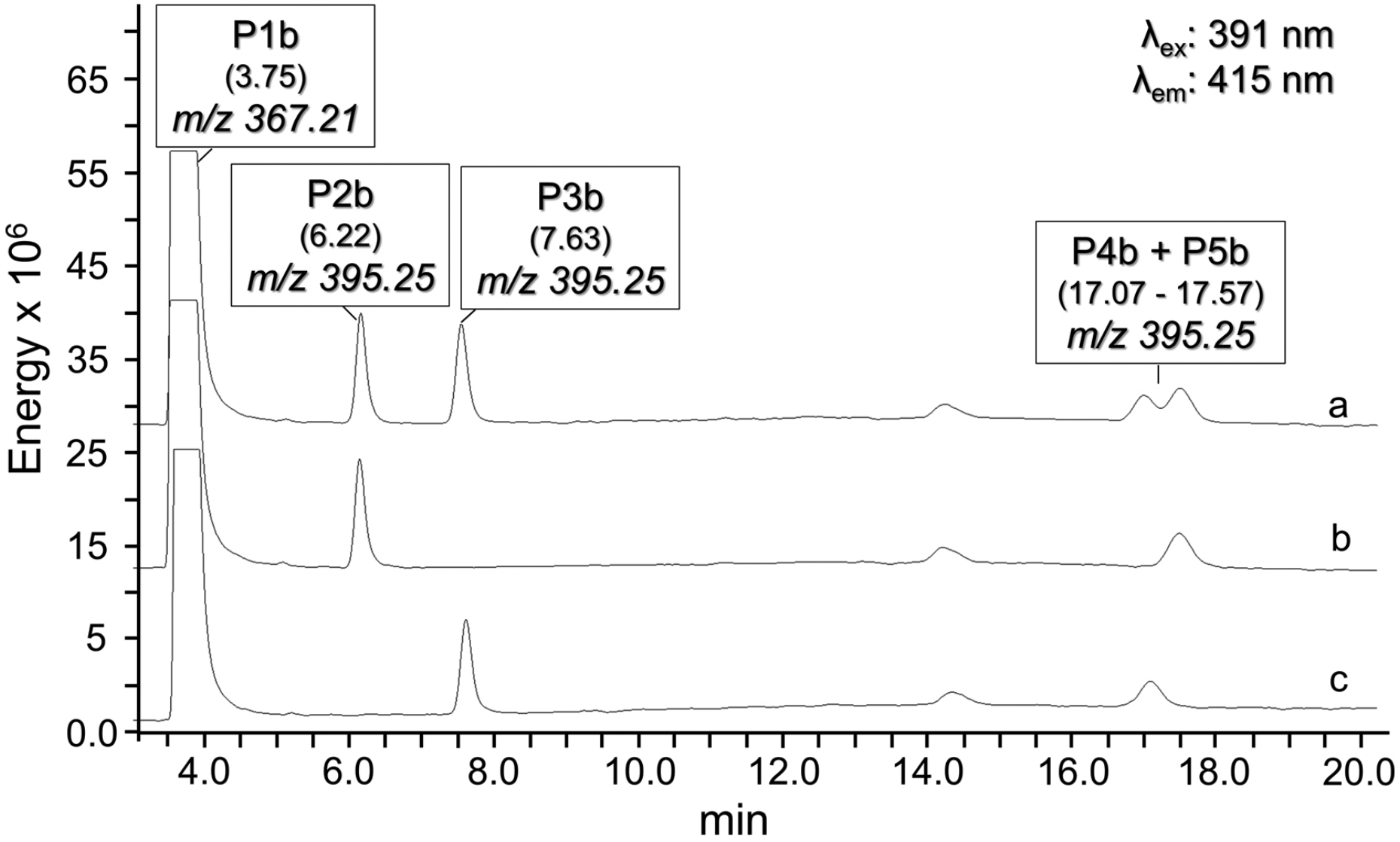
nm

Figure 4


P1b
(3.75)
m/z 367.21
P2b
(6.22)
m/z 395.25
P3b
(7.63)
m/z 395.25
P4b + P5b
(17.07 - 17.57)
m/z 395.25
λex: 391 nm
λem: 415 nm
a
b
c
Energy x 10^6
0.0
5
15
25
35
45
55
65
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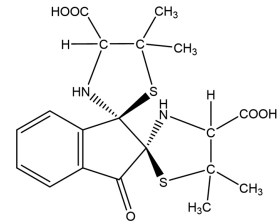
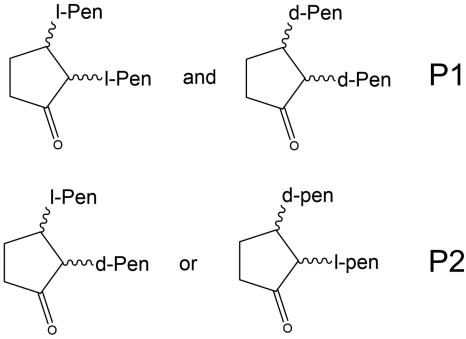
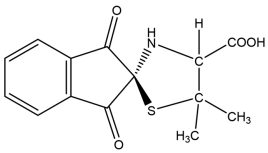
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
min

Figure 5

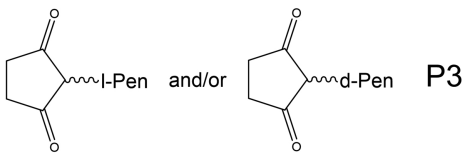


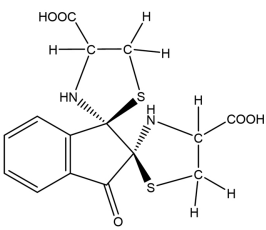

Double-spirane (Pen/Pen) *m/z* 423.41



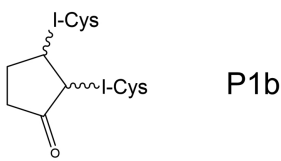



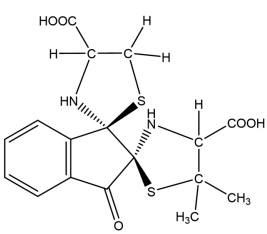

Single-spirane (Pen) *m/z* 292.34




Double-spirane (Cys/Cys) *m/z* 367.21




Mixed double-spirane
(Cys/Pen; Pen/Cys) *m/z* 395.25

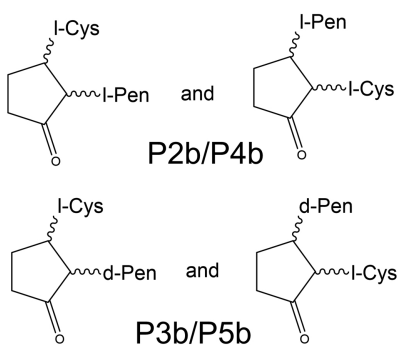
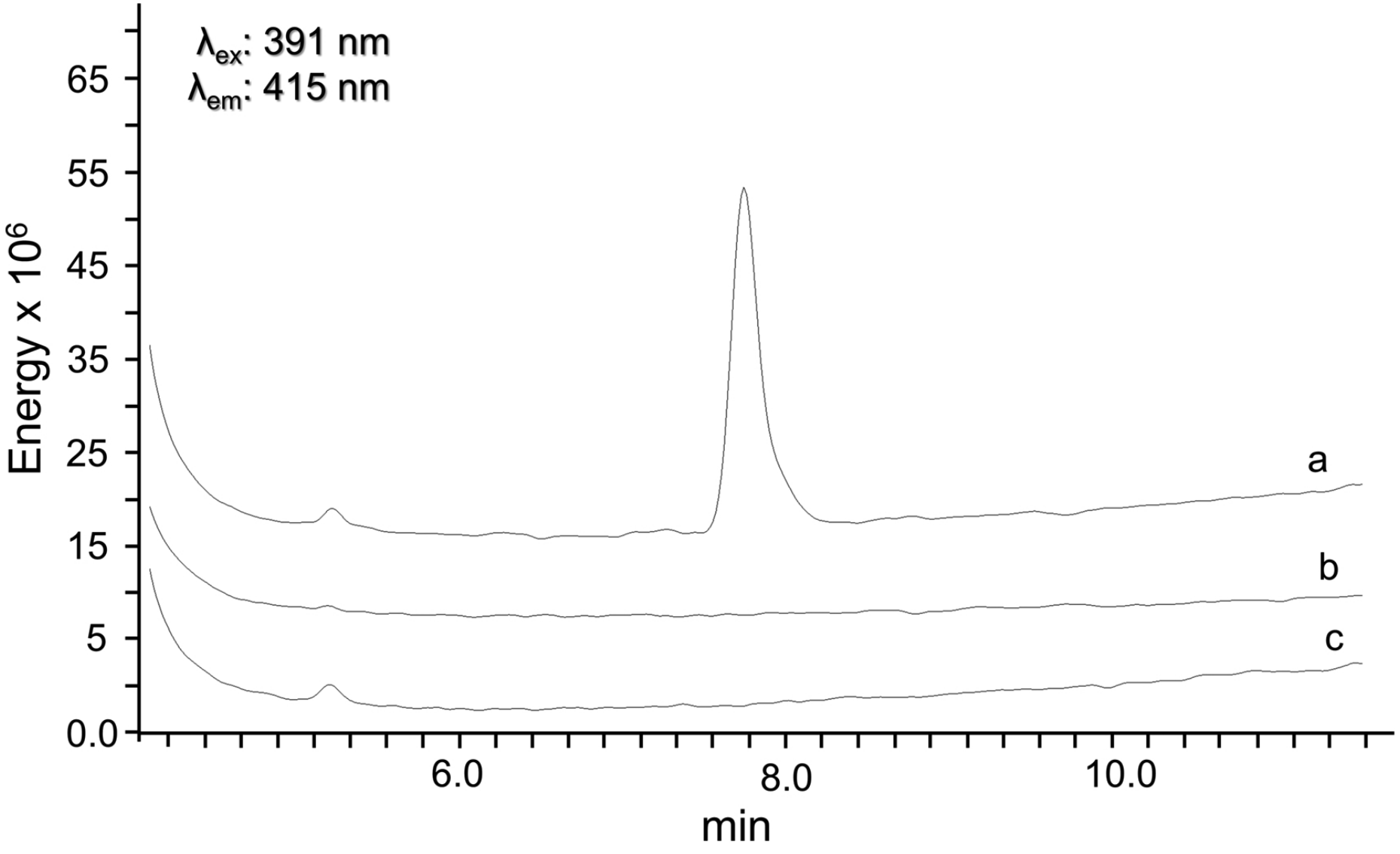



Figure 6


λex: 391 nm
λem: 415 nm
Energy x 10^6
65
55
45
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6.0
8.0
10.0
min
a
b
c

Figure 7