Dithiolane linked thiorhodamine dimer for Hg^{2+} recognition in living cells†

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Thiorhodamine-based chemodosimeter **A**, a disulfide linked dimer, was designed for Hg^{2+} recognition by virtue of the strong affinity of mercury for sulfur. Spectroscopic results reveal that chemodosimeter **A** exhibits real-time responses, and high sensitivity and selectivity for Hg^{2+} in comparison to other cations. These properties are mechanistically ascribed to the transfer from rhodamine spirolactam to the thiazoline-derived open-ring rhodamine *via* Hg^{2+} induced desulfurization. The *in vitro* recognition of Hg^{2+} in living cells pretreated with **A** was examined, showing that the concentration of Hg^{2+} that could be imaged reaches the safety limit for human beings.

Introduction

Mercury is the third most frequently found and second most common toxic heavy metal in the list of the Agency for Toxic Substances and Disease Registry (ATSDR) of the U. S. Department of Health and Human Services.[1,2] The extreme toxicity of mercury and its derivatives results from its high affinity for thiol groups in proteins and enzymes, leading to the dysfunction of cells and consequently causing health problems.[3] The health concerns over exposure to mercury have motivated the exploration of selective and efficient methods for the monitoring of mercury in biological and environmental samples.[4,5] A number of fluorescent chemosensors with selectivity for Hg^{2+} have been reported including conjugated polyelectrolytes,[6] foldamers,[7] biomolecules,[8] and small molecules.[9] Of these, fluorescence probes derived from biocompatible substrates offer a promising approach for the *in vitro* and *in vivo* monitoring of mercury due to their low costs and easy cellular uptakes.[5]

Rhodamine derivatives exhibit high molar absorption coefficients and high fluorescence quantum yields at longer wavelengths (>500 nm) when in ring-open conformations, but their spirolactam structures are colorless and nonfluorescent. More recently, several rhodamine-based probes were reported, in which the signal was transduced either through simply opening the spirolactam ring upon mercury binding[10] or through chemodosimetric reactions of the thiocarbonyl activated by the strong affinity of mercury for sulfur.[11] However, most of them have cross-sensitivities toward other metal cations or delayed responses. For example, these chemodosimetric sensors take one minute or several minutes to complete the ring-opening reaction. In the present work, a rhodamine-based chemodosimeter **A** with a disulfide linker (Scheme 1) was designed on the basis of the extreme affinity of sulfur for mercury. It shows the features of unique selectivity and real-time responses to mercury over other cations in aqueous solution. Compound **A** was obtained from the reaction of compound **B** (Scheme 1) and P_2S_5/HMDO (hexamethyl disiloxane) in refluxing $CHCl_3$ (yield: 19%). Compound **B** was synthesized from the simple reaction of rhodamine B acyl chloride and cystamine dihydrochloride in a yield of 55.6%. The detailed synthetic procedures and characterizations are described in the Experimental Section.

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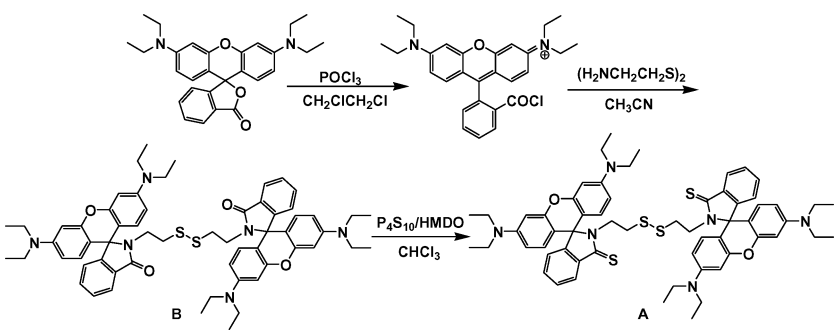
† Electronic supplementary information (ESI) available: IR, ^{1}H NMR, ^{13}C NMR and HRMS spectra of **A**, **B**, and **C**; the reversibility of compound **A** and **B** with DETA; the effect of different pH on the fluorescence response of **A** and **A** + Hg^{2+}; confocal images of living HK-2 cells in the presence of compound **A**. See DOI: 10.1039/b815956d



Scheme 1 The synthesis of compounds **A** and **B**.

Results and discussion

Hg^{2+} recognition in aqueous solution

Compounds **A** and **B** exhibit selectivity for Hg^{2+} among a series of metal cations, such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Li^{+}, K^{+}, Na^{+}, Cu^{+}, Ag^{+} and Fe^{3+}. However, it takes several hours to observe the fluorescence enhancement for compound **B** upon the addition of Hg^{2+} (Fig. 1a). Additionally, the fluorescence change of compound **B** was reversed upon addition of diethylenetriamine (DETA) (Fig. S1, ESI†). This returning phenomenon elicits that the spectral response of compound **B** to Hg^{2+} is likely due to the chelation-induced ring-opening of rhodamine spirolactam,[10,12] rather than other possible reactions. However, the formation of complex [**B**-Hg]$^{2+}$ might decrease the electrophilicity of Hg^{2+}, which is needed to open the rhodamine spirolactam, consequently slowing down the fluorescence response.

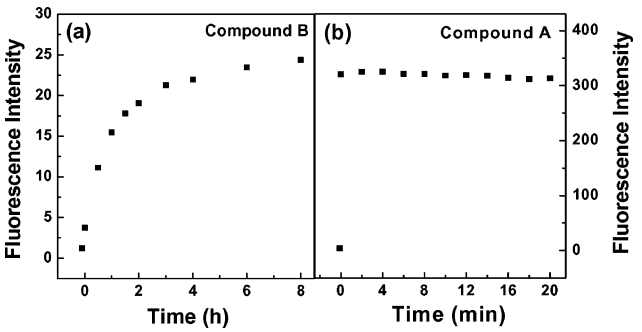


Fig. 1 Time response curves of compound **B** (10 μM) (a) and compound **A** (5 μM) (b) to the addition of 5 equiv. of Hg^{2+} in an ethanol-water (v/v = 80/20) solution. λ_{ex} = 510 nm, λ_{em} = 580 nm.

Significantly different from compound **B**, the solution color and fluorescence changes of **A** were observable upon the addition of Hg^{2+} within 10 seconds (Fig. 1b). This feature of real-time response to mercury is particularly important in practical application. In addition, the solutions of compound **A** and **A** + Hg^{2+} remain stable in a wide pH range from 4 to 9 (Fig. S2, ESI†).

As shown in Fig. 2, the growth of an absorption peak at 560 nm and a maximum emission at 580 nm were observed upon gradual addition of Hg^{2+}. The plot of fluorescence intensity of compound **A** against the concentration of added Hg^{2+} is depicted in Fig. 2b (insert), and exhibits a good linear correlation in the concentration range from 0.1 μM to 10 μM (R^2 = 0.99875). The fluorescence intensity of compound **A** increased with increasing concentration of Hg^{2+} to 2 equiv., but remained unchanged with further addition of Hg^{2+} (Fig. 3). The Job's plot (Fig. 3, insert) was conducted when [**A** + Hg^{2+}] = 10^{-5} M, and the turn point clearly appears at 0.66, indicating the 1:2 stoichiometric ratio of compound **A** to Hg^{2+}.

In order to evaluate the selectivity of compound **A** for Hg^{2+} among a series of metal cations, such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, Li^{+}, K^{+}, Na^{+}, Cu^{+}, Ag^{+} and Fe^{3+}, the fluorescence spectra of compound **A** (5 μM) in an ethanol-water (v/v = 80/20) solution upon addition of various metal caions are depicted in Fig. 4a. Apparently, a significant fluorescence enhancement of compound **A** (~50 times) was observed only when Hg^{2+} was added, in comparison to that seen by adding other cations (<5 times). Moreover, the Hg^{2+} induced

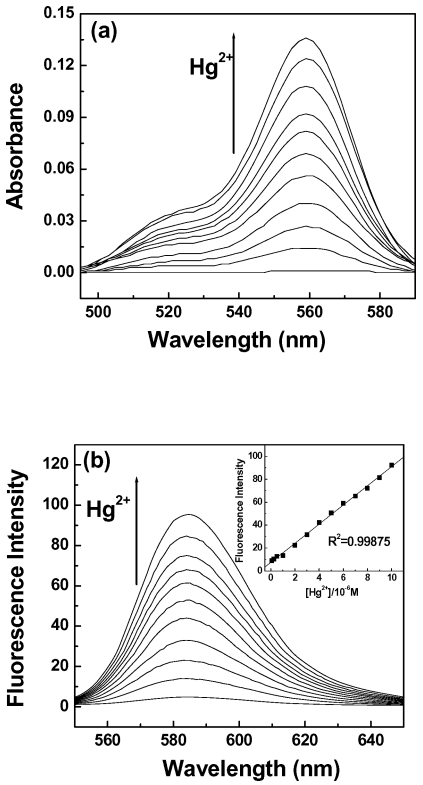


Fig. 2 UV-Vis absorption (a) and fluorescence emission (b) spectra of **A** (5 μM) in an ethanol-water (80/20, v/v) solution upon addition of Hg^{2+} in concentrations of 0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 μM. Insert: the plot of fluorescence intensity of compound **A** (5 μM) upon additions of Hg^{2+} from 0.1 to 10 μM in ethanol-water (v/v = 80/20) solution. λ_{ex} = 510 nm, λ_{em} = 580 nm.

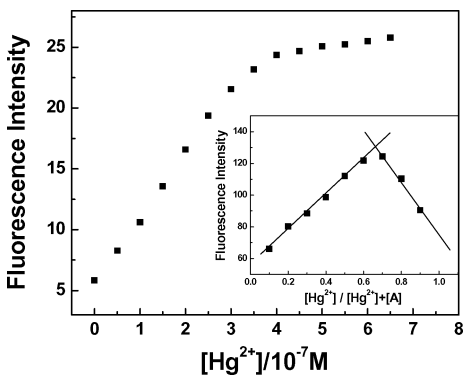


Fig. 3 The titration curve of **A** (0.2 μM) upon addition of Hg^{2+} in an ethanol-water (v/v: 80/20) solution. Insert: Job's plot for the complex of **A** and Hg^{2+} in ethanol-water (v/v = 80/20) solution. [**A**] + [Hg^{2+}] = 10^{-5} M. λ_{ex} = 510 nm, λ_{em} = 580 nm.

fluorescence enhancement was not affected by the presence of alkali or alkaline-earth metals, such as Ca^{2+}, Mg^{2+}, Li^{+}, K^{+}, Na^{+}, and other transition metals like Cu^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{+}, Ag^{+} and Fe^{3+}. As shown in Fig. 4b, the fluorescence intensity of compound **A** in the presence of other cations upon the addition of Hg^{2+} was approximately invariant. All together the results illustrate that the selectivity and responsive time of **A**

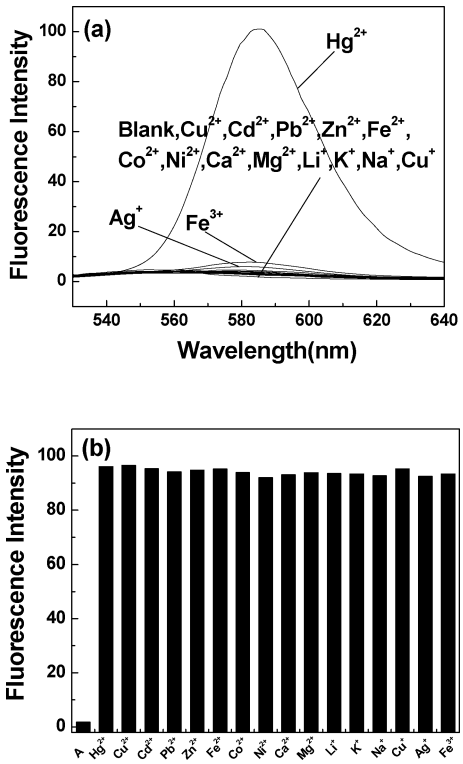


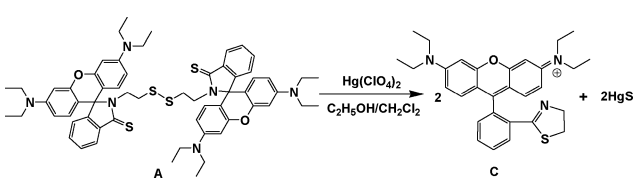
Fig. 4 (a) The fluorescence spectra of **A** (5 μM) upon addition of 10 μM of Hg^{2+} and 50 μM of various other metal ions in an ethanol-water (v/v = 80/20) solution. (b) Fluorescence response of **A** (5 μM) to 10 μM of Hg^{2+} in an ethanol-water (v/v = 80/20) solution containing 50 μM of various metal ions. $\lambda_{ex} = 510$ nm, $\lambda_{em} = 580$ nm.

are remarkably improved for Hg^{2+} recognition in comparison to **B** possibly due to the transformation of the C=O in **B** to C=S in **A**.

Rationalization

To rationalize the sensitivity and selectivity of compound **A** for Hg^{2+}, an excess of DETA was added into a compound **A** solution in the presence of Hg^{2+} (Fig. S3, ESI†). Unlike the phenomenon observed in the same experiment conducted with compound **B**, the fluorescence spectra of compound **A** remained unchanged after the excellent chelating agent of transition metals (DETA) was added. The result implies an irreversible nature for the desulfurization, which was also observed in those chemodosimeters typical for Hg^{2+}.[11,13]

To characterize the desulfurization induced by Hg^{2+}, the reaction of compound **A** with Hg^{2+} in ethanol/CH_2Cl_2 was conducted. The molecular structure of the unique reaction product was characterized by IR, ^{1}H NMR, ^{13}C NMR and HRMS (ESI†) to be compound **C** (Scheme 2) with a high fluorescence quantum yield ($\Phi_f = 0.53$ in reference to rhodamine B).[14] Similarly to other Hg^{2+}



Scheme 2 The synthesis of compound **C**.

induced cyclizations,[11] a rhodamine derivative with a thiazoline structure was formed.

Intracellular imaging of Hg^{2+}

Taking into account the advantages of real-time response, extremely high sensitivity and selectivity of compound **A** for Hg^{2+}, compound **A** was used for *in vitro* Hg^{2+} detection in living cells. After HK-2 cells were incubated with 10 μM of compound **A** for 30 min at 37 °C, and then treated with different concentrations of Hg^{2+} from 0.1, 1, 10, to 20 μM, their fluorescence images were taken by confocal microscopy. As shown in Fig. 5, these confocal images display Hg^{2+}-concentration dependence: the stronger fluorescence images of HK-2 cells are those treated with the higher concentrations of Hg^{2+}. The imaged intracellular Hg^{2+} concentration was as low as 0.1 μM (0.1 ppm) (Fig. 5a), the limit of safe concentration for human beings. Under the same experimental conditions, no fluorescence was imaged in HK-2 cells incubated with compound **A** only (Fig. S4a, ESI†). Based on these results, any concerns about fluorescence emission possibly arising from the enzymatic ring-opening of the thiorhodamine spirolactam in living cells can be ruled out. In addition, the bright field transmission image of the HK-2 cell was displayed in Fig. S4b (ESI†).

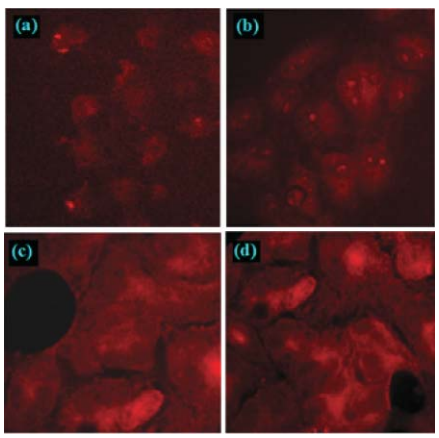


Fig. 5 Confocal images of HK-2 cells incubated with **A** (10 μM) for 30 min at 37 °C and then treated with (a) 0.1 μM, (b) 1 μM, (c) 10 μM and (d) 20 μM of Hg^{2+} for 30 min.

Conclusions

In conclusion, a thiorhodamine-based chemodosimeter **A** with a disulfide linker was synthesized and characterized. The experimental results show that compound **A** exhibits real-time responses, and an extremely high sensitivity and unique selectivity for Hg^{2+}, which are mechanistically ascribed to the transfer from thiorhodamine spirolactam to the thiazoline-derived open-ring rhodamine **C** through Hg^{2+} induced desulfurization. The utilization of compound **A** for the monitoring of mercury levels in living cells was thus examined, and showed that the concentration of Hg^{2+} that could be imaged, reached the safety limit for human beings. The reported chemodosimeter **A** containing a disulfide linker might find its application in mercury recognition in biological systems.

Experimental section

Materials and methods

UV-vis absorption and fluorescence spectra were recorded in a Hitachi U-3010 absorption spectrometer and a Hitachi F-4500 fluorescence spectrometer, respectively. NMR spectra were recorded on a Bruker-400 (400 MHz). Mass spectroscopy was measured on either a Finnigan GC-MS 4021 or a Bruker Paltonics FlexAnlysis BA060655 MS-spectrometer instrument. Cationic compounds such as $LiClO_4$, $NaClO_4$, $KClO_4$, $AgNO_3$, CuI, $Mg(ClO_4)_2$, $Ca(ClO_4)_2$, $Pb(ClO_4)_2$, $Fe(ClO_4)_2$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $Cu(ClO_4)_2$, $Zn(ClO_4)_2$, $Cd(ClO_4)_2$, $Hg(ClO_4)_2$ were purchased from Aldrich, and were used as received. Rhodamine B base was analytical grade, purchased from Beijing Chemical and recrystallized once before use. Dry 1,2-dichloroethane and acetonitrile were distilled from these refluxing solvents with CaH_2. Ethanol for spectra detection was HPLC reagent without fluorescent impurity and H_2O was deionized water. Flash chromatography was carried out on silica gel 60 (200–300 mesh; Qingdao Haiyang Chemical Co., Ltd).

All experiments were carried out in an ethanol/water (v/v = 80/20) solution except when specifically described. The stock solution of **A** or **B** was prepared in ethanol/CH_2Cl_2 (v/v = 8/2) (1.0×10^{-3} M), stored at −25 °C and thawed in the dark before use. The cationic solutions were prepared in ethanol/water (v/v = 80/20) with concentrations of 1.0×10^{-2} M for fluorescence and UV-vis measurements. To a quartz cell (1 cm of optical path length) filled with 2 mL of **A** or **B** was added the stock solution of cations dropwise using a micro-syringe. The volume of these added cationic stock solutions was less than 100 μL to leave the concentration of **A** or **B** unchanged. All fluorescence spectra were recorded at 25 °C under excitation of 510 nm. The maximum fluorescence intensities at 580 nm were analyzed against the concentrations of added cations.

Cell culture

HK-2 cells (gifted from the center of cells, Peking Union Medical College) were cultured in culture media (DMEM/F12 supplemented with 10% FBS, 50 unit/mL penicillin, and 50 μg/mL of streptomycin) at 37 °C in a humidified incubator. HK-2 cells were seeded in a 6-well plate at a density of 104 cells per well in culture media. After 24 h, the cells were incubated with 10 μM of **A** in culture media for 20–30 min at 37 °C. After carefully washing with PBS to remove free **A**, the cells pretreated with **A** were then incubated with $HgCl_2$ in different concentrations in culture media for another 30 min at 37 °C. These cells were imaged using confocal fluorescence microscopy (excitation light source: Green; Olympus IX 71 S 8F-2).

Synthesis

Compound B. To a solution of rhodamine B base (1.0 g, 2.3 mmol) in 1,2-dichloroethane (15 mL), was added phosphorus oxychloride (0.75 mL, 8.2 mmol) dropwise over 5 min under stirring. The mixture solution was refluxed for 4 hours. After the reaction mixture was cooled down to r.t., the solvent was removed under reduced pressure to obtain rhodamine B acyl chloride, which was directly used in the next step. The crude rhodamine B acyl chloride (2.3 mmol) was dissolved in acetonitrile (30 mL) and added dropwise to a solution of cystamine dihydrochloride (0.3 g, 1.3 mmol) in dry acetonitrile (15 mL) containing 2.0 mL of triethylamine over 1~1.5 h in an ice bath. After 8 h, the solvent was removed under reduced pressure and the residue was washed with water. The purple residue was dried in a vacuum oven and purified by column chromatography (using CH_2Cl_2/methanol = 20:1 as eluent). The first pink band was collected to afford **B** (0.64 g, yield: 55.6%). IR (KBr pellet, cm^{-1}): 3078, 2969, 2928, 1693, 1614, 1515, 1229, 1118, 819, 785, 756, 699. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.17 (24H, t, J = 7.0 Hz), 2.27 (4H, q), 3.29 (4H, m), 3.35 (16H, q, J = 7.0 Hz), 6.28 (4H, d), 6.38 (4H, s), 6.45 (4H, d), 7.07 (2H, m), 7.43 (4H, m), 7.90 (2H, m). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 12.7, 35.9, 40.0, 44.4, 65.0, 97.9, 105.5, 108.0, 122.7, 124.0, 128.1, 128.8, 131.8, 132.4, 149.0, 153.7, 167.5. TOF-HRMS $[M]^+$: m/z, found 1000.4746, calcd 1000.4743.

Compound A. Compound **B** (100 mg, 0.1 mmol) was dissolved in 10 mL of $CHCl_3$ and added to a mixture of P_4S_{10} (32 mg, 0.07 mmol) and HMDO (0.2 mL, 0.94 mmol) in 10 mL of $CHCl_3$. The reaction mixture was refluxed for 4 hours under a N_2 atmosphere. After the removal of $CHCl_3$, the residue was purified by chromatography (CH_2Cl_2/petroleum = 4:1 as eluent) to give **A** (20 mg, yield: 19%). IR (KBr pellet, cm^{-1}): 2968, 2967, 1615, 1516, 1397, 1220, 1118, 818, 770. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.12 (24H, t, J = 6.5 Hz), 2.39 (4H, m), 3.28 (16H, m), 3.54 (4H, m), 6.26 (12H, m), 7.09 (2H, m), 7.49 (4H, m), 8.12 (2H, m). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 12.7, 29.8, 35.0, 44.5, 73.6, 98.1, 103.4, 108.2, 123.5, 124.9, 128.7, 132.4, 138.6, 149.3, 150.1, 153.5, 190.0. MALDI-TOF-MS found $[M + H]^+$: m/z, 1033.462, calcd 1033.44.

Compound C. Compound **A** (103 mg, 0.1 mmol) was dissolved in CH_2Cl_2/ethanol (1:1, 20 mL). To the solution of **A**, was added $Hg(ClO_4)_2 \cdot 3H_2O$ (227 mg, 0.5 mmol). After stirring for 10 min, the solvent was removed under reduced pressure and the residue was purified by column chromatography (using ethyl acetate/ethanol = 3/1 as eluent). The red band was collected and the solvent was removed under vacuum to afford **C** (15.6 mg, yield: 16.1%). IR (KBr pellet, cm^{-1}): 2970, 2926, 1589, 1412, 1338, 1181, 1074, 822, 683. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.33 (12H, t, J = 7.12 Hz), 3.21 (2H, t, J = 8.4 Hz), 3.63 (8H, q, J = 7.16 Hz), 4.00 (2H, t, J= 8.4), 6.80 (2H, m), 6.89 (2H, d), 7.14 (2H, d), 7.27 (1H, m), 7.68 (2H, m), 7.95 (1H, m). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 12.8, 34.6, 46.2, 65.5, 96.79, 113.7, 113.9, 130.3, 130.5, 130.7, 130.9, 131.6, 133.4, 155.5, 157.8 166.3. ESI-HRMS $[M]^+$: m/z, 484.2433 found, calcd 484.2417.

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