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Recognition of Hg^{2+} Using Diametrically Disubstituted Cyclam Unit

Prasenjit Mahato, Amrita Ghosh, Sukdeb Saha, Sandhya Mishra, Sanjiv K. Mishra, and Amitava Das*

Central Salt and Marine Chemical Research Institute, (CSIR), Bhavnagar -364002, Gujarat, India

Received July 23, 2010

A newly synthesized 1,4,8,11-tetraazacyclotetradecane derivative (**L**), functionalized with a diazo moiety as the reporter functionality, is found to bind specifically to Hg^{2+} with an associated change in color that could be visually detected. With biologically benign β-CD, it forms an inclusion complex (**L**·**2**β-**CD**), which shows a much higher solubility in water, and this helps in developing a more intense color on binding to Hg^{2+} in a CH_3CN-HEPES buffer medium. The nontoxic nature of **L** was checked with the living cells of a Gram negative bacterium, *Pseudomonas putida*. Further, experiments revealed that these two reagents could be used as staining agents for the detection of Hg^{2+} present in this microorganism.

Introduction

Mercury is a dangerous toxin that has become widespread throughout our environment and can pose a serious threat to the food chain and thus human health. Coal-fired power plants, waste incinerators, chloro-alkali plants, gold mining,[1] and sources like oceanic and volcanic emission[2] add a substantial quantity of mercury in various forms to the environment. Atmospheric oxidation of mercury vapor leads to the generation of water-soluble Hg^{2+} ions that deposit onto land or into water and is assimilated by microorganisms, which convert some of it to methylmercury, a potent neurotoxin linked to many cognitive and motion disorders.[3] Bioaccumulation of this occurs as bigger animals eat smaller ones.[4] While attempts are being made to curb the mercury emissions substantially from medical and municipal waste incinerators,[5] very little effort has been made to curb the mercury pollution from coal-fired power plants.[6] Thus, the development of new methods for the selective detection and visualization of Hg^{2+} ions in chemical and biological systems is critically important, and significant research efforts have been devoted to improve mercury detection and recognition.[7,8] Current industrial approaches rely on costly, time-consuming methods like atomic absorption/emission or inductively coupled plasma mass spectroscopy, which are not very convenient or handy for "in-the-field" detection. A number of selective Hg^{2+} sensors have been devised using either redox,[9] chromogenic,[10] or fluorogenic[11] changes. Further, chromogenic sensors that allow visual detection have an edge over others owing to obvious simplicity in the recognition process; its naked eye detection offers qualitative and quantitative information without using expensive equipment. However, such examples

*To whom correspondence should be addressed. Telephone: +91 278 2567760 [672]. Fax: +91 278 2656762. E-mail: amitava@csmcri.org.

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of the reversible recognition of Hg^{2+} are relatively scarce compared to those which can detect Hg^{2+} ions in aqueous solution by chemodosimetry.[8a−e,12] Thus, despite widespread interest and recent advances, there are only a few examples of chromogenic probes that bind specifically and reversibly to Hg^{2+} in the presence of all other metal ions, like alkali, alkaline earth, common transition metal (more specifically metal ions like Pb^{2+} and Cu^{2+}), and lanthanide ions, and are capable of detection of Hg^{2+} in biological samples.[13] Thus, the design and synthesis of such new synthetic molecules remains an outstanding challenge. Recent reports suggest that nitrogen binding sites are a good choice for the selective recognition of heavy metal ions such as Cd^{2+}, Pb^{2+}, and Hg^{2+}.[14] More recently, Ho and his co-workers have shown that 8,8′-(1,4,10,13-tetrathia-7,16-diazacyclooctadecane-7,16-diyl)-bis(methylene)diquinolin-7-ol could be used for the recognition of Hg(II), present in micromolar concentrations in the presence of various cations like Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, and Al^{3+}, while some of the metal ions are reported to interfere with detection if they are present in relatively much higher concentrations.[14] This leaves us with the opportunity to design and synthesize a suitable receptor with a softer base as a coordinating unit, which may allow us to achieve a better specificity over a wider range of metal ions including Cd^{2+}, Cu^{2+}, and lanthanide ions without sacrificing much of its binding ability to the target Hg^{2+} ion. With this aim, we could synthesize a diametrically disubstituted 1,4,8,11-tetraazacyclotetradecane (cyclam) derivative, functionalized with 4-(4-dimethylamino)phenyl azobenzene as the reporter moiety, which has shown remarkable specificity towards Hg^{2+} in the presence of a wide range of interfering metal ions in aqueous solutions (including Cd^{2+}, Pd^{2+}, Cu^{2+}, and all lanthanide ions). We are reporting herein its synthesis, binding studies, and [3]pseudorotaxane formation in the presence of β-cyclodextrin (β-CD). Further, results of the experimental studies reveal that [3]pseudorotaxane formation improves the solubility of the receptor in aqueous solutions and thereby the colorimetric detection phenomena.

Experimental Section

1,4,8,11-Tetraazacyclotetradecane, 4-(4-dimethylaminophenylazo)-benzenesulphonyl chloride, β-cyclodextrin, $Hg(ClO_4)_2$, $Cu(ClO_4)_2$, $Zn(ClO_4)_2$, $Ni(ClO_4)_2$, $Fe(ClO_4)_2$, $Pb(ClO_4)_2$, $Cd(ClO_4)_2$, $Cr(ClO_4)_3$, $Ca(ClO_4)_2$, $Co(ClO_4)_2$, $NaClO_4$, $KClO_4$, $Mg(ClO_4)_2$, $CsClO_4$, $Ba(ClO_4)_2$, $Ce(NO_3)_3\cdot 6H_2O$, $Eu(NO_3)_3\cdot 5H_2O$, $Er(NO_3)_3\cdot 5H_2O$, $Nd(NO_3)_3\cdot 6H_2O$, $Tb(NO_3)_3\cdot 5H_2O$, $Yb(NO_3)_3\cdot 5H_2O$, $Pr(NO_3)_3\cdot 6H_2O$, $Ho(NO_3)_3\cdot 6H_2O$, $Lu(NO_3)_3\cdot xH_2O$, $Dy(NO_3)_3\cdot xH_2O$, $Gd(NO_3)_3\cdot 6H_2O$, $Sm(NO_3)_3\cdot 6H_2O$, $La(NO_3)_3\cdot 6H_2O$, and $Tm(NO_3)_3\cdot 5H_2O$ were obtained from Sigma-Aldrich and were used as received. All of the other reagents used were procured from S. D. Fine Chemicals, India. Acetonitrile, chloroform, methanol (AR; Merck, India), and ethanol (Spectrosol; Spectrochem, India) were used as solvents. HPLC-grade water (Merck, India) was used for experiments and spectral studies. ESI-MS measurements were carried out on Waters QTof-Micro instrument. Microanalysis (C, H, N) was performed using a Perkin-Elmer 4100 elemental analyzer. FTIR spectra were recorded as KBr pellets using a Perkin-Elmer Spectra GX 2000 spectrometer. ^{1}H and ^{31}PNMR spectra were recorded on a Bruker 500 MHz FT NMR machine (model: Avance-DPX 500). Electronic spectra were recorded with a Shimadzu UV-3101 PC/Varian Cary 500 Scan UV−vis−NIR Spectrophotometer. The images of the *Pseudomonas putida* cells were viewed under a normal light microscope (AXIO IMAGER−Carl Zeiss).

Synthesis of L. 1,4,8,11-Tetraazacyclotetradecane (cyclam; 247 mg, 1.23 mM) was dissolved in dry chloroform (40 mL) in a 250 mL round-bottomed flask. A solution of 4-(4-dimethylaminophenylazo)-benzenesulphonyl chloride (800 mg, 2.46 mM) and triethyl amine (Et_3N) (1 mL) in 60 mL of dry chloroform was added to the above solution under ice-cold conditions with stirring. Then, the resulting mixture was allowed to stir at room temparature for 10 h, and then it was further refluxed for 1 h. The mixture was cooled to room temperature and evaporated to dryness using a rotary evaporator. Then, the crude product was purified by column chromatography using silica gel as a stationary phase and a chloroform/methanol mixed solvent (98:2, v/v) as the eluent. Isolated yield of the compound **L** (yield was calculated on the basis of the starting compounds): 48%. ^{1}H NMR (500 MHz, $CDCl_3$, $SiMe_4$, J (Hz), δ (ppm)): 7.96 (4H, d, J = 8.5, Ar−H_{16}), 7.92−7.88 (8H, m, Ar−$H_{17,20}$), 6.76 (4H, d, J = 9.0, Ar−H_{21}), 3.38 (4H, br, $H_{2,9}$), 3.21 (12H, br, $H_{3,5,7,10,12,14}$), 3.13 (12H, s, H_{23} −N(CH_3)$_2$), 2.26 (4H, br, $H_{6,13}$). ^{13}C NMR (DMSO-d^6, 500 MHz, $SiMe_4$, δ (ppm)): 155.05, 153.19, 142.56, 135.29, 128.90, 125.48, 122.32, 111.54, 48.11, 47.84, 47.63, 45.80, 39.8, 26.43. ESI-MS (+ve mode): m/z 775.93 (100%) (M^+). Calcd for $C_{38}H_{50}N_{10}O_4S_2$: 776.36. Elemental Anal. Calcd: C, 58.74; H, 6.75; N, 18.03. Found: C, 58.9; H, 6.8; N, 18.1.

Synthesis of L_1. A total of 100 mg (0.31 mM) of 4-(4-dimethylamino-phenylazo)-benzenesulphonyl chloride was added to 15 mL of pyrrolidine (excess) under an inert atmosphere and heated to reflux for 24 h. Then, the reaction mixture was evaporated to

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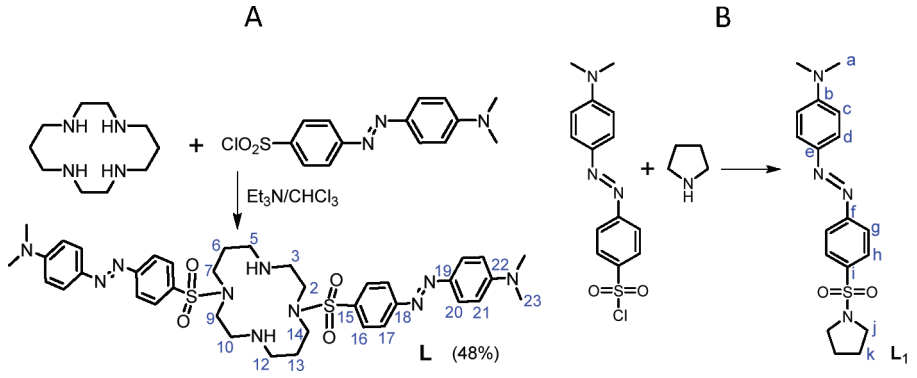
Scheme 1. Methodology Adopted for the Synthesis of (A) **L** and (B) $\mathbf{L_1}$



dryness. The crude product was recrystallized from methanol to obtain $\mathbf{L_1}$ in pure form with 85% yield (yield was calculated on the basis of the starting compounds). ^{1}H NMR (500 MHz, CD_3OD, $SiMe_4$, J (Hz), δ (ppm)): 7.93 (2H, d, J = 8.4, Ar−H_h), 7.87−7.8 (4H, m, Ar−$H_{d,g}$), 6.83 (2H, d, J = 9, Ar−H_c), 3.27−3.32 (4H, t, H_j(−CH_2)), 3.1 (6H, s, H_a −N(CH_3)$_2$), 2.03−1.96 (4H, m, H_k(−CH_2)). ESI-MS (+ve mode): m/z 359.18 (5%) ($M^+ + H^+$). Calcd for $C_{18}H_{22}N_4O_2S$: 358.46. Elemental Anal. Calcd: C, 60.3; H, 6.2; N, 15.6. Found: C, 60.4; H, 6.1; N, 15.5.

Spectrophotometric Titration. Evaluation of the Association Constant for L·Hg^{2+} Formation. A 11.5 μM solution of **L** in an acetonitrile−HEPES buffer (pH 7.2, 2:3 (v/v)) was prepared and stored under dark conditions. These solutions were used for all spectroscopic studies after appropriate dilution. A 16.62 mM solution of $Hg(ClO_4)_2$ was prepared using the same solvent composition. A solution of **L** was used for spectroscopic titrations after appropriate dilution, and the effective final concentration was adjusted to 2.3 μM, while the final analyte (Hg^{2+}) concentration for titration was varied from 0 to 13.3 mM.

Evaluation of the Association Constant for [L·2β-CD] Formation. A 21.15 μM solution of **L** in an acetonitrile−HEPES buffer (pH 7.2, 2:3 (v/v)) was prepared and stored under dark conditions. These solutions were used for all spectroscopic studies after appropriate dilution. A 3.64 mM solution of β-CD was prepared using the same solvent composition. A solution of **L** was further diluted for spectroscopic titrations, and the effective final concentration was adjusted to 4.23 μM, while the effective [β-CD] was varied from 0 to 2.91 mM.

Evaluation of the Association Constant for Hg^{2+}·[L·2β-CD]. A 34.5 μM solution of **L** in an acetonitrile−HEPES buffer (pH 7.2, 1:9 (v/v)) was prepared in the presence of 83 μM β-CD and stored under dark conditions. This solution was used for all spectroscopic studies after appropriate dilution. A 6.03 mM solution of Hg^{2+} was prepared using the same solvent acetonitrile−HEPES buffer (pH 7.2, 1:9 (v/v)) composition. A solution of **L·2β-CD** was further diluted for spectroscopic titrations. The effective final concentration was adjusted to 6.9 μM, and the [Hg^{2+}] concentration was varied from 0 to 2.41 mM.

Calculations for the Binding Constants Using Spectrophotometric Titration Data. The following equation was used for the nonlinear least-squares analysis to determine the association constants as well as the stoichiometries for the formation of the complexes **L·Hg^{2+}** and **Hg^{2+}·L·2β-CD**:[15]

$$A = (A_0 + A_{\lim} K_n C_M^n)/(1 + K_n C_M^n) \quad (1)$$

where A_0, A, and $A_{\lim}$ are the absorbances of free **L**, **L** present in the form of **L·Hg^{2+}** in the complex, and **L** in presence of excess amounts of Hg^{2+} ions where the absorbance reaches a limiting value. C_M is the metal ion concentration, and n is the stoichiometry of the complex formed between the ligand and metal ion.

The following equations were used for the nonlinear least-squares analysis to determine the association constants of the complexes formed between **L** and β-CD:[16]

$$\frac{1}{A - A_0} = \frac{1}{K_1(A_1 - A_0)} \times \frac{1}{[\beta\text{-CD}]_0} + \frac{1}{A_1 - A_0} \quad (2)$$

$$\frac{1}{A - A_0} = \frac{1}{(A_2 - A_0)K_1K_2} \times \frac{1}{[\beta\text{-CD}]_0^2} + \frac{1}{(A_2 - A_0)} \quad (3)$$

A_0 and A_1 denote the absorbance of free **L** and **L** in the complex, and K_1 is the association constant for 1:1 complexation. A_2 is the absorbance in the complex, and K_2 is the association constant for 1:2 complexation.

Biological Study. *Pseudomonas putida* (*P. putida*) was cultured in King's B (KB) medium (peptone, 20 g; glycerol, 15 g; K_2HPO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; distilled water, 1000 mL; pH 7.2). The cells were harvested and vortexed for making the homogeneous suspension in sterile distilled water.

The cultured cells were first exposed to different concentrations of Hg^{2+} (6 ppb to 3.75 ppm) in a ethanol/HEPES buffer (2:3, v/v; pH 7.2) for 30 min at 25 °C. Then, free Hg^{2+} that was present in the solution was removed through centrifugation. This process was repeated after adding ~5 mL of a ethanol−HEPES buffer (pH 7.2, 2:3 (v/v)) to remove traces of Hg^{2+} ions that may still be present on the microorganism surfaces in order to avoid the background color while recording optical microscope images. The centrifuged bacterial cells were finally exposed to **L** (12 μM) in a 2:3 ethanol/HEPES buffer (v/v; pH 7.2). The treated bacterial cells were monitored through an optical microscope (Carl Zeiss; 40 X), and microscopic images of bacterial cells in the absence (control) and presence of Hg^{2+} were observed for comparison. For checking the suitability of **L·2β-CD** as the staining agent, similar experiments were repeated; **L·2β-CD** was used instead of **L**. The centrifuged bacterial cells were finally exposed to **L·2β-CD** (11.5 μM **L** in the presence of 10 molar equivalent of β-CD) in an ethanol−HEPES buffer (pH 7.2, 1:9 (v/v)) and were monitored through an optical microscope (Carl Zeiss; 40 X), and microscopic images of bacterial cells in the absence and presence of Hg^{2+} were observed for comparison (Figures S5 and S6 in the Supporting Information).

Live cultures of *Pseudomonas fluorescens* and *Pseudomonas putida* were used for our studies. *Pseudomonas putida* showed better tolerance to high concentrations than *Pseudomonas fluorescens*. Earlier report revealed that the *Pseudomonas putida*

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strain could tolerate heavy metals up to a concentration of 3 mM/L.[17]

To check the nontoxic nature of the reagents **L** towards *Pseudomonas putida*, cultures (500 μL) of *P. putida* were incubated in the absence (control) and presence of 5.0 mg of the respective dye for 2 h in an incubator at 34 °C. The control and the other culture treated with dye (**L**) were separately poured on three different King's B agar plates, and then these two plates were incubated at 34 °C for 24 h. The growth of *Pseudomonas putida* was observed on both agar plates to examine whether **L** had any toxic effect towards the viability of this microbe (Figure S7 in the Supporting Information).

Results and Discussion

Synthesis of the new sensor molecule (**L**, Scheme 1A) was achieved by reacting 1,4,8,11-tetraazacyclotetradecane (cyclam) and 4-(4-dimethylamino-phenylazo)-benzenesulphonyl chloride in an appropriate molar ratio. After necessary purification by column chromatography, **L** was achieved in pure form, which was confirmed by various analytical data. $\mathbf{L_1}$ was isolated in pure form after recrystallization of the crude product that was obtained through the reaction of the sulphonyl chloride fragment with excess pyrrolidine (Scheme 1B).

UV−vis spectra for **L** (CH_3CN−aq. HEPES buffer, 2:3 (v/v), pH 7.2) show an intraligand $\pi-\pi^*$ charge transfer band with λ_{max} at 469 nm (Figure 1A), which predominantly involves π^{NMe_2}[HOMO]- and $\pi^{[N=N]^*}$[LUMO]-based frontier orbitals. Analogous absorption band maxima for $\mathbf{L_1}$ appeared at 452 nm in a CH_3CN−aq. HEPES buffer medium (2:3 (v/v), pH 7.2). A greater electron withdrawing ability of the substituted azamacrocyclic moiety in **L** than that of the pyrrolidine moiety in $\mathbf{L_1}$ is expected to inflict a narrower HOMO−LUMO gap for **L**, which can be accounted for by its observed red-shifted absorption maxima for **L** (Figure S8 in the Supporting Information).

This binding ability of **L** towards various metal ions (all alkali, alkaline earth, all common transition metal, and lanthanide ions) was checked by recording the electronic spectra of **L** in the absence and presence of respective metal ions. No change in spectra for **L** could be observed in the presence of any one of these cations, except Hg^{2+} (Figure S9 in the Supporting Information). Electronic spectra of **L** in a CH_3CN−aq. HEPES (2:3, v/v) buffer solution (pH 7.2) with varying [Hg^{2+}] are shown in Figure 1A. A new spectral band with maxima at 494 nm developed (red shift of ~25 nm) along with the appearance of an isosbestic point at 466 nm. This suggests that the new complex $\mathbf{Hg^{2+}\cdot L}$ was formed and existed in equilibrium with **L**. Simultaneously, a color change of the solution from dark yellow to pink-red could be visually detected, while no such color change could be observed for all of the other metal ions added (Figure 1B). The observed red shift could be attributed to the more favored ICT process for $\mathbf{Hg^{2+}\cdot L}$ as compared to that for **L**.

Previous experimental and theoretical reports on Hg^{2+}-cyclam′ (cyclam′ is 1,8-dimethyl-4,11-tetraazacyclotetradecane) complexes suggest an R,S,R,S configuration at the N-stereocenter as the most preferred conformation in solution.[18,19] Thus, it would not be unreasonable to propose an R,S,R,S configuration at the N-stereocenter for **L**, when bound to Hg^{2+} in solution (Scheme 2). This conformation further favors the enhancement of the dipolar vector for **L** on coordination to Hg^{2+} (Scheme 2) and is also expected to contribute to the observed red shift in the absorption maxima on coordination to the Hg^{2+} ion. A more significant red shift in this ICT band maximum for $\mathbf{Hg^{2+}\cdot L}$ (474 to 491 nm), as compared to **L** (441 to 450 nm), on increasing the solvent polarity from THF to CH_3CN further supports this configuration (Figure S10 in the Supporting Information). Spectrophotometric and ESI-MS studies revealed that the binding of Hg^{2+} to **L** followed a 1:1 stoichiometry (Figure S11 in the Supporting Information).

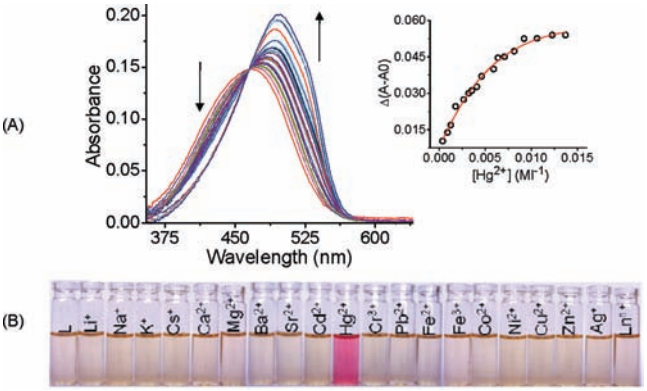

Figure 1. (A) Absorbance spectra of **L** (2.3 μM) with varying [Hg^{2+}] (0−13.3 mM) in CH_3CN−aq. HEPES buffer (0.01 M, pH 7.2; 2:3, v/v). Inset: Least square plot of $\Delta[A - A_0]$ vs [Hg^{2+}]. (B) Change in color of **L** (5 μM) in a CH_3CN−aq. HEPES buffer (0.01 M, pH 7.2; 2:3, v/v) in the presence of various metal ions (2.5 mM).

Electronic spectra for $\mathbf{L_1}$ (2.45×10^{-5} M) were also checked in the absence and presence of a large excess of Hg^{2+} (0.02 M) in a CH_3CN−aq. HEPES (2:3, v/v) buffer solution (pH 7.2; Figure S12 in the Supporting Information). The absorption maximum for $\mathbf{L_1}$ was found to be 452 nm, and an insignificant shift of 4 nm (λ_{max} = 456 nm) was observed in the presence of a large excess of Hg^{2+} (Figure S12 in Supporting Information). This tends to nullify the possibility of the binding of Hg^{2+} either to the $-NMe_2$ or to the azo- functionality of $\mathbf{L_1}$. Thus, this observation confirms that the observed red shift of absorption maxima for **L** in the presence of Hg^{2+} is due to the coordination of Hg^{2+} to the N atoms that belong to the azamacrocycle moiety. The noncoordinating or at best weakly coordinating nature of $\mathbf{L_1}$ to the Hg^{2+} ion was also evident in the ^1H and ^{13}C NMR spectra recorded in the absence and presence of excess Hg^{2+} ions (vide infra).

The possibility of any involvement of the sulphonyl (S═O) functionality in **L** for a probable Hg^{2+}−O═S sulphonyl-type coordination could be nullified on the basis of the FTIR studies. The FTIR spectrum for **L** shows bands at 1364 and 1134 cm^{-1}, which are characteristic for asymmetric and symmetric stretching frequencies, respectively, for the sulphonyl group. These band positions remained practically unchanged and appeared at 1365 and 1133 cm^{-1} even in the presence of 33 molar equivalents of Hg^{2+} (Figure S13 in the Supporting Information). Thus, one would rule out any possibility of the involvement of a sulphonyl moiety in coordination to Hg^{2+}, as for such a situation an appreciable shift in IR frequency for the sulphonyl functionality would be expected.

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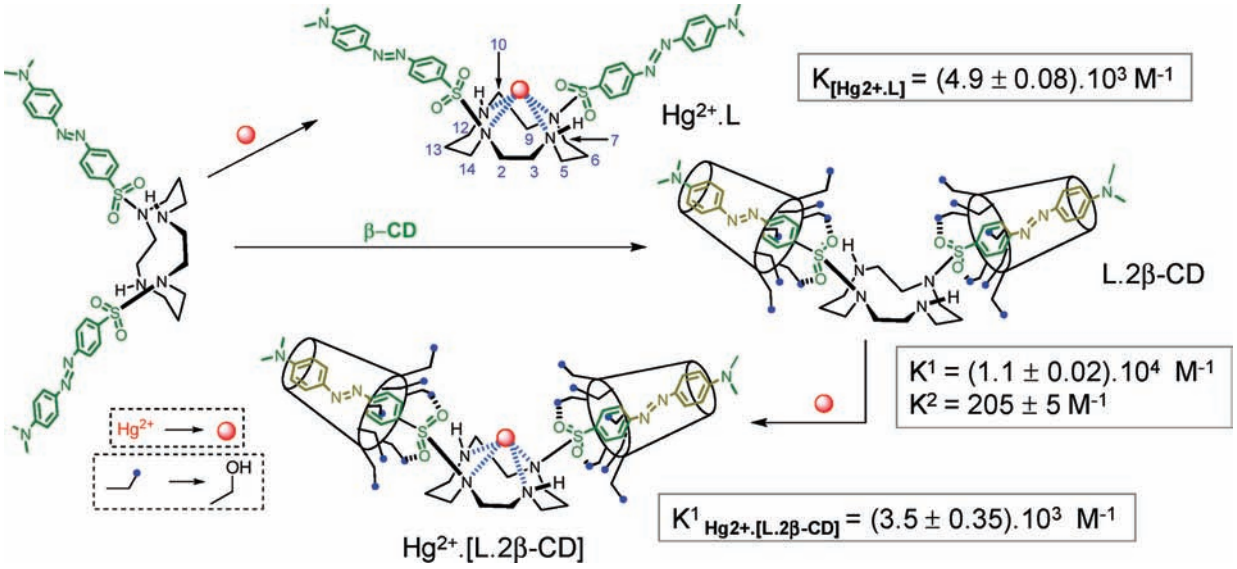
(18) (a) Enoki, O.; Imaoka, T.; Yamamoto, K. *Org. Lett.* **2003**, *5*, 2547. (b) Liang, X.; Sadler, P. J. *Chem. Soc. Rev.* **2004**, *33*, 246. (c) Hunter, T. M.; McNae, I. W.; Simpson, D. P.; Smith, A. M.; Moggach, S.; White, F.; Walkinshaw, M. D.; Parsons, S.; Sadler, P. J. *Chem.—Eur. J.* **2007**, *13*, 40.
(19) Hrobarik, P.; Kaupp, M.; Riedel, S. *Angew. Chem., Int. Ed.* **2008**, *47*, 8631.

Scheme 2. Schematic Presentation of **L**, $\mathbf{Hg^{2+}\cdot L}$, $\mathbf{L\cdot 2\beta\text{-}CD}$, $\mathbf{Hg^{2+}\cdot[L\cdot 2\beta\text{-}CD]}$



The association constant for the complex ($\mathbf{Hg^{2+}\cdot L}$) formation was evaluated from systematic titration using the nonlinear least-squares method and was found to be $(4.91 \pm 0.08) \times 10^3\ M^{-1}$.[15] A relatively lower association constant value (K_{assoc}) obtained for $\mathbf{Hg^{2+}\cdot L}$, compared to the classical cyclam−Hg^{2+} complex, may be ascribed to the attachment of two electron-withdrawing sulphonyl moieties to the two N atoms of the cyclam unit.[14c] The presumably soft nature of two out of four N atoms of **L** accounts for the unusual specificity towards Hg^{2+}. The lowest detection limit for Hg^{2+} was found to be 28 ppb under these experimental conditions (Figures S14 and S15 in the Supporting Information). On adding an CH_3CN−HEPES (2:3, v/v; pH = 7.2) buffer solution of Na_2EDTA (15.5 mM) to $\mathbf{Hg^{2+}\cdot L}$ (3.8 mM, CH_3CN−aq. HEPES buffer, (2:3, v/v; pH 7.2), the absorption band with λ_{max} at 490 nm (characteristic for $\mathbf{Hg^{2+}\cdot L}$) disappeared, and spectra for **L** with a λ_{max} of 469 nm were restored (Figure S16 in the Supporting Information) with a change in color from pink-red to pale yellow. This confirmed the reversible binding nature of **L** towards Hg^{2+}. Further, the specificity towards the Hg^{2+} ion was established, as no change in absorbance at 494 nm for $\mathbf{Hg^{2+}\cdot L}$ was observed (Figure S17 in the Supporting Information) in the presence of a 10 molar excess of each of the metal ions studied (Na^+, K^+, Cs^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Sr^{2+}, Pb^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Pr^{3+}, Eu^{3+}, Er^{3+}, Nd^{3+}, Dy^{3+}, Yb^{3+}, Ce^{3+}, Ho^{3+}, Lu^{3+}, Tm^{3+}, Tb^{3+}, La^{3+}, Sm^{3+}, and Gd^{3+}) or in the presence of a mixture of all of these metal ions, while each of these metal ions were present in a 1.2 molar excess relative to that of [Hg^{2+}]. Thus, experimental studies revealed that the reagent **L** could be used for specific recognition of Hg^{2+} in the presence of a wide range of metal ions that belong to groups I and II, common transition metal ions, and all lanthanide ions in a mixed aqueous−acetonitrile medium.

^{1}H NMR Studies. ^{1}H NMR spectra for **L** and $\mathbf{L_1}$ were recorded in $CDCl_3$ and CD_3OD, respectively; assignments of the individual H atoms of these compounds are provided in the Experimental Section. ^{1}H NMR spectra for **L** and $\mathbf{L_1}$ were also recorded in the presence of varying [Hg^{2+}] in DMSO-d_6 and DMF-d_7 solvents, respectively. Gradual upfield shifts were observed for all aromatic H atoms (H_{16}, H_{17}, H_{20}, H_{21}) on the successive addition of Hg^{2+}, and eventually upfield shifts of about 18 to 20 Hz were observed on complete binding to Hg^{2+} (97 molar equivalent). However, these upfield shifts were more significant for aliphatic H atoms (Figures S18 and S19 in the Supporting Information). Such upfield shifts upon metal ion binding are not very uncommon in the literature.[8a,20] A shift of ∼28 Hz was observed for H_7 and H_{14} hydrogen atoms on the binding of **L** to Hg^{2+}. Signals for H_5 and H_{12} became very broad on coordination to Hg^{2+}; however, the shift was >36 Hz. The shift for H_6 and H_{13} atoms was also ∼36 Hz, while that for H_{23} was 25.8 Hz, which was comparable to the shift observed for other aromatic H atoms in **L**. The relative shift data in ^{1}H NMR spectra tend to nullify the possibility of coordination through the $-NMe_2$ of **L** and, thus, it was not considered. All of these tend to indicate that **L** was bound to Hg^{2+} through four nitrogens of the tetrazamacrocyclic component, and this accounted for the higher upfield shift for the aliphatic H atoms of the macrocyclic ring. To ascertain this, we recorded the ^{1}H NMR spectra for $\mathbf{L_1}$ in the absence and presence of Hg^{2+} (Figure S20 in the Supporting Information). The shift of signals for H_h, H_d, and H_g of $\mathbf{L_1}$ on the addition of excess (100 molar equivalents) Hg^{2+} was ∼0.2 Hz, a value within the limit of experimental error, while that for H_c was 1.6 Hz. This shift in the $\Delta\delta$ value for H_j is 0.4 Hz. Considering these minor shifts for $\mathbf{L_1}$, as compared to the shifts observed for **L** on binding to Hg^{2+}, led us to confirm that neither the aromatic part nor the N_{NMe_2} part of **L** or $\mathbf{L_1}$ was involved in coordination to Hg^{2+}.

^{13}C NMR Spectra. ^{13}C NMR spectra for **L** and $\mathbf{L_1}$ were recorded in absence and presence of Hg^{2+} and are shown in Supporting Information (Figures S21, S22 and S23 in Supporting Information). Upfiled shifts in the range of 225−625 Hz were observed for aliphatic C-atoms of the tetraazamacrocyclic ring in **L**; while those for different aromatic C-atoms lies within the range of 25 − 85 Hz. Similar studies with $\mathbf{L_1}$ revealed shifts of 0.5−8.5 Hz.

(20) Shiraishi, Y.; Maehara, H.; Ishizumi, K.; Hirai, T. *Org. Lett.* **2007**, *9*, 3125.

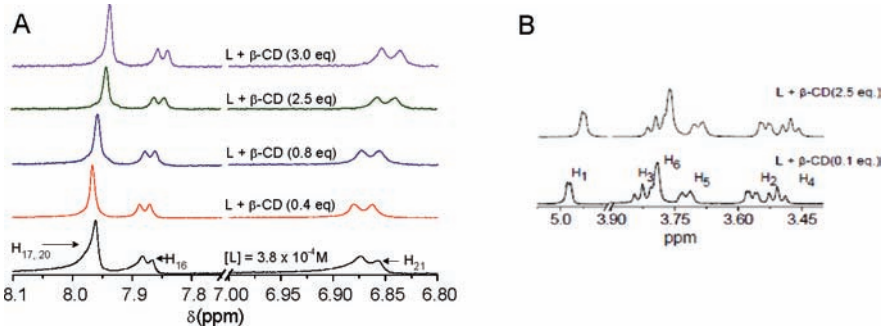


Figure 2. (A) Partial ^{1}H NMR spectra of **L** (8 mM) with varying [β-CD] (0−15.2 mM; in CD_3CN-D_2O, 1:1, v/v). (B) Partial ^{1}H NMR spectra of **L·2β-CD** for δ = 3.45−5 ppm, a region where **L** does not have any resonance signal.

This comparison clearly shows that even if $\mathbf{L_1}$ had any interaction with Hg^{2+}, the extent of interaction was unimportant as compared to that between **L** and Hg^{2+}. Thus, any possibility of the observed binding of Hg^{2+} to **L** through the aromatic moiety can be ignored on the basis of the results of the ^{1}H and ^{13}C NMR spectral studies.

Inclusion Complex Formation with β-CD. The limited solubility of the ligand **L** in water did not allow us to check the recognition process in either a pure aqueous solution or a pure HEPES buffer medium. Derivatives of 4,4′-bipyridine, like (E)-1,2-di(pyridin-4-yl) diazene, analogous to **L** are known to form stable [3]pseudorotaxane-type inclusion complexes with β-cyclodextrin (β-CD), and the formation of such a complex is expected to enhance the solubility of these bipyridyl derivatives in water.[21] We studied the formation of such an inclusion complex (**L·2β-CD**, Scheme 2) by ^{1}H NMR and electronic spectroscopy (Figures 2A, B and 3). A gradual upfield shift for all aromatic hydrogen atoms (H_{16}−H_{21}) of **L** (8 mM) was observed on the addition of increasing amounts of β-CD, and these shifts were more prominent for H_{17} and H_{20} (∼ 11 Hz, Figure 2A). For the same study, changes in the positions for aliphatic hydrogen atoms of the tetraaza macrocyclic component were almost negligible (∼ 4 Hz). Upfield shifts of ∼15 Hz were also observed for the hydrogens (H^1−H^6) of the β-CD moiety (Figure 2B). Similar upfield shifts were also reported for an analogous [3]pseudorotaxane formation with 4,4′-bipyridine derivatives and β-CD.[21,22] The presence of the deshielding effect due to diamagnetic anisotropy of the benzenoid moiety of the included azo compound could account for such upfield shifts. Thus, the ^{1}H NMR study tends to confirm the inclusion complex formation, while the 1:2 binding stoichiometry was confirmed by ESI-MS spectra recorded for **L** (8.7 μM in CH_3CN/H_2O of 1:1, v/v) in the presence of excess β-CD (1.14 mM; Figure S24 in the Supporting Information). A mass spectral signal (m/z) at 3044.9 was observed, and this corresponds well with the 1:2 adduct formation, i.e, a [3]pseudorotaxane formation (Scheme 2). The binding process of β-CD and the azobenzene guest is expected to be driven by favorable enthalpy changes, which could attribute to the significant contribution of hydrophobicity, hydrogen bonds, and van der Waals interactions, and C−H···π interactions between host and guest molecules. Inclusion complex formation between **L** and β-CD was also studied spectrophotometrically. A little red shift of 3 nm was observed in the electronic spectra for **L** on the addition of excess β-CD (Figure 3). A very close examination revealed the appearance of two consecutive isosbestic points at 387 and 544 nm, with an increase in [β-CD]. This tends to suggest the presence of two different equilibrium processes.

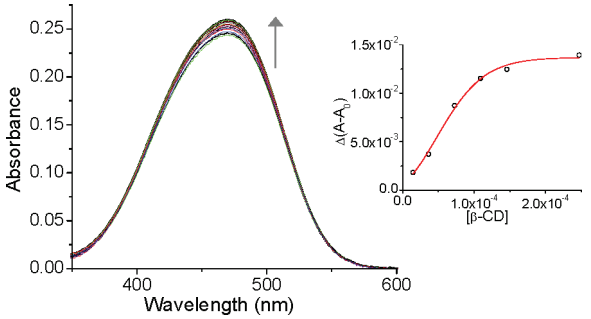


Figure 3. Absorbance spectra of **L** (4.23 μM) with varying [β-CD] (0−0.247 mM) in a CH_3CN−aq. HEPES buffer (0.01 M, pH 7.2; 2:3, v/v). Inset: plot of change in absorbance ($A - A_0$) of **L** against varied concentrations of β-CD.

On the basis of the previous reports,[21,22] it may be argued that various noncovalent interactions,[23] which account for the stability of the [3]pseudorotaxane (**L·2β-CD**), could lower the energy gap between the frontier orbitals and were reflected in the little red-shifted absorption band. Further, the apolar interior of β-CD requires less reorganization to accompany the electronic transition(s) in **L·2β-CD**, than in the solvent surroundings for **L**.[24] Although small, this may also contribute to lowering the energy of activation for optical electron transfer. Interestingly, least-squares analysis[16] of the spectrophotometric titration data revealed two equilibrium processes with binding constants of (1.1 ±

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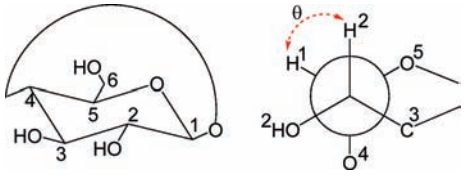
Scheme 3. Schematic Representation of (A) Pyranose Structure and (B) Gauche Conformation of β-CD with θ as the Dihedral Angle Involving Two Vicinal Hydrogen Atoms, H^1 and H^2, of the Pyranose Ring



$0.02) \times 10^4$ (K_1) and 205 ± 5 (K_2) M^{-1}. Two different equilibrium constants could account for the stepwise formation of **L·β-CD** and **L·2β-CD**. However, it is not possible to completely rule out the possibility of two-step inclusion reactions of β-CD and azobenzene that involve a hasty inclusion in the first step and slower solvational and/or conformational changes after inclusion in the second step.[21i] A similar explanation was offered for the observed two-step inclusion process involving a related sulphonamide derivative of the azobenzene compound and α-CD on the basis of detailed ^{1}H NMR and variable pressure kinetic studies.[21i,25] In the present situation, after initial inclusion of **L** in the β-CD cavity, a possible polar interaction between the $-SO_2-$ moiety of the sulphonamide fragment and $-CH_2OH$ functionality of the β-CD larger rim could induce conformational changes of the host cavity and thus a new equilibrium process with some minor spectral shifts.

Further support for the conformational change(s) of the host β-CD cavity was available in the detailed ^{1}H NMR studies. ^{1}H NMR spectra of β-CD (3.5 mM) and β-CD in the presence of **L** (7.0 mM) were recorded in CD_3CN-D_2O (1:1, v/v). A critical examination, a comparison of these two ^{1}H NMR spectra, and an evaluation of the coupling constants for the interaction between vicinal H^1 and H^2 hydrogen of the pyranose structure suggested a definite change in the coupling constant, $^3J_{12}$, for the β-CD moiety (Scheme 3). This change in coupling constant value (Δ^3J_{12}) can be used to calculate the change in dihedral angle (θ) involving two vicinal H^1 and H^2 atoms of the pyranose ring of the host β-CD moiety on forming an inclusion complex with **L**. The modified Karplus equation was used for the calculation of θ is shown and this can be expressed by the following equation:[21k,25]

$$^3J_{12} = (6.6 - 1.0\cos\theta + 5.6\cos 2\theta) \times \{1 - \Sigma_{i=1}^{4}(f_i\Delta X_i)\} \quad (4)$$

where $\Delta X = X - X_H$ represents the difference in electronegativity between a substituent and hydrogen (the electronegativities used are $X_H = 2.1$, X_{Or}, $X_{OCH_3} = 3.3$, and $X_{-C-O-} = 2.5$, and the electronegativity factor $f_i = 0.1$).[21k] When these electronegativity values and $^3J_{12}$ values are used before and after the inclusion of **L** in the β-CD cavity, $\Delta\theta = 1$ was evaluated (Table S1 in the Supporting Information). The $\Delta\theta = 1$ value for the present study is less than what was reported for the formation of an inclusion complex between an analogous sulphonamide derivative of a diphenyl azo fragment and α-CD,[21i] which is understandable considering the larger cavity diameter of β-CD. This

(25) Durette, L.; Horton, D. *Org. Magn. Res.* **1971**, *3*, 417.

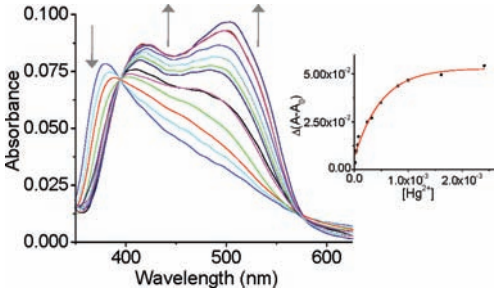


Figure 4. Spectrophotometric titration of **L·2β-CD** ([**L**], 6.9 μM; [β-CD], 16.6 μM) with varying [Hg^{2+}] (0−2.41 mM) in a CH_3CN−aq. HEPES buffer (0.01 M, pH 7.2 ;1:9, v/v). Inset: plot of $\Delta(A - A_0)$ vs [Hg^{2+}].

conformational change may have some influence on the observed spectral changes and contribute to the observed second equilibrium; however, it is difficult to visualize and predict the extent of the effect of this conformational change on the overall changes in electronic spectra.

The affinity of **L·2β-CD** towards Hg^{2+} was examined. It was observed that in the presence of β-CD, **L** was found to be soluble in a mixed solvent medium with an even higher proportion (1:9, v/v) of water in a CH_3CN−water/aq. HEPES buffer medium. Systematic changes in the spectral pattern for **L·2β-CD** were recorded with increasing amounts of added [Hg^{2+}] in a CH_3CN/aq. HEPES buffer medium (1:9, v/v; Figure 4), and the absorption maximum for **L·2β-CD** at 380 nm was found to decrease with a concomitant increase in absorbance at 420 and 502 nm. Appearances of two simultaneous isosbestic points at 394 and 576 nm revealed the presence of only two interacting components and an equilibrium, which are involved in this binding process. The binding stoichiometry and association constant for the formation of the complex, **Hg^{2+}·[L·2β-CD]**, was evaluated using the nonlinear least-squares method and found to be 1:1 and $3.52 \times 10^3 \pm 35$ M^{-1}, respectively.[15] A little lower binding affinity of **L·2β-CD**, as compared to **L**, towards Hg^{2+} under identical experimental conditions could account for the higher solvation and thereby the slower diffusion of **L·2β-CD** compared to **L**. The lowest detection limit for Hg^{2+} was found to be 34.4 ppb under these experimental conditions (Figure S25 in the Supporting Information). The more intense absorption band tail in the longer wavelength region was attributed to an even more prominent color change for **Hg^{2+}·[L·2β-CD]** than that for **Hg^{2+}·L** (Figure S26 in the Supporting Information).

This prompted us to explore the possibility of using these as colorimetric reagents for the detection of the uptake of Hg^{2+} by microbes. *Pseudomonas putida* is known to be resistant towards Hg^{2+} and to adsorb Hg^{2+} at the cell surface and cell membrane.[26] Thus, we have used cells of *Pseudomonas putida* to check for such a possibility. We have used pre-exposed (0−45 min at 25 °C) bacterial cell (*Pseudomonas putida*, Figure 5; Figures S5 and S6 in the Supporting Information) suspensions of the Hg^{2+} ion ([Hg^{2+}] was 6 ppb) in an aqueous medium for studies.

Each culture was exposed separately to a 12 μM solution of **L** and **L·2β-CD** (11.5 μM) and was viewed through

(26) (a) Clark, D. L.; Weiss, A. A.; Silverj, S. *J. Bacteriol.* **1977**, *132*, 186. (b) Rezaee, A.; Derayat, J.; Mortazavi, S. B.; Yamini, Y.; Jafarzadeh, M. T. *Am. J. Environ. Sci.* **2005**, *1*, 102. (c) Nascimento, A. M. A.; Chartone-Souza, E. *Genet. Mol. Res.* **2003**, *2*, 92.

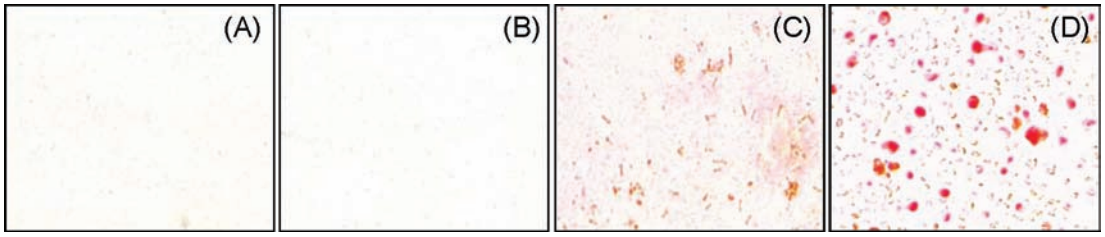


Figure 5. Light microscope images of (A) control cells of *Pseudomonas putida*, (B) cells exposed to Hg^{2+} (6 ppb), (C) cells exposed to an aqueous solution of Hg^{2+} (6 ppb) for 45 min and then to **L** (12 μM, ethanol−aq. HEPES buffer (0.01 M, pH 7.2; 2:3 (v/v)), and (D) cells exposed to an aq. solution of Hg^{2+} (6 ppb) for 45 min and then to **L·2β-CD** (11.5 μM **L** in the presence of 10 molar equivalents of β-CD, ethanol−aq. HEPES buffer (0.01 M, pH 7.2) (1:9, v/v)).

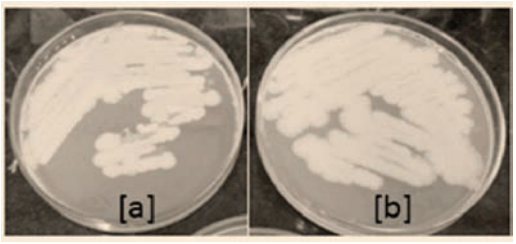


Figure 6. Culture of *Pseudomonas putida* on a King's B agar plate (a) in the absence and (b) in the presence of **L** after incubation at 34 °C for 24 h.

an optical microscope (Carl Zeiss; 40 X). Respective images of the control and cells in the presence of Hg^{2+} and other reagents are shown in Figure 5. This clearly revealed that the staining of the cells (pre-exposed to 6 ppb of Hg^{2+} ion) could be achieved by **L** or **L·2β-CD**, while, staining was more prominent for **L·2β-CD**. It is evident from our study that microorganisms should not be overlooked when studying metal contaminants in the soil. It is worth mentioning that a [Hg^{2+}] of 6 ppb is much lower than the international norm for STLC regulatory levels (0.2 ppm).[27]

(27) Soluble Threshold Limit Concentration (STLC). Chapter 11, Article 3, Identification and Listing of Hazardous Waste; California Code of Regulations, Title 22, **2005**.

An isolated experiment revealed that **L** was nontoxic to the living bacterial cells used. Luxuriant growth was observed for both the King's B agar plates (incubated at 34 °C for 24 h) having a culture of *Pseudomonas putida* in the absence and presence of the added dye (**L**) (Figure 6). It establishes that the culture is viable with the ligand, and the receptor molecule **L** does not show any harmful effect on the growth of the bacteria.

Thus, we could demonstrate that **L** could be used for the detection of Hg^{2+} adsorbed on the cell surface of microorganisms such as *Pseudomonas putida*. The extent of staining and visual detection by optical microscopy could be further improved upon by using its [3]pseudorotaxane form (**L·2β-CD**) in the presence of biologically benign reagents like β-CD. Such an example of *in vivo* detection of Hg^{2+}, in a lower organism, using a nontoxic colorimetric reagent is not common in the literature.

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Supporting Information Available: Characterization data of **L**, $\mathbf{L_1}$, and **L·2β-CD**; spectral details; and biological studies. This material is available free of charge via the Internet at http://pubs.acs.org.