


**Final Report**

**Project Title** Development of New Endotoxin Sensors Based on Novel Anti-sepsis Scaffold

**By** Dr.Kriangsak Khownium

**Aug 31st, 2020 (finish date)**

**Contract No.** MRG5680030

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| Researcher | Institute |
| --- | --- |
| **Dr. Kriangsak Khownium** | **King Mongkut's Institute of Technology Ladkrabang** |



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# 1. Abstract

## 1.1 Abstract

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Project Title : Development of New Endotoxin Sensors Based on Novel Anti-sepsis Scaffold

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**Abstract:**

The development of a more robust and more specific fluorescent probe is highly desirable for time-critical diagnosis and treatment of sepsis patients. We envision that *bis*-cationic nature of the terephthalaldehyde-*bis*-guanylhydrazone scaffold would be a good template to develop a series of small molecule sensors with effective selectivity for lipopolysaccharide (LPS, endotoxin) detection through electrostatic interactions with *bis*-phosphate portion of lipid A. Bispyrenyl terephtalaldehyde-bis-guanylhydrazone (**BPTG**) fluorescent chemosensor with two fluorogenic pyrene units has been designed and synthesized for lipopolysaccharide (LPS) detection through excimer emission ‘‘turn-on’’. It shows a dose-dependent increase of fluorescent intensity in aqueous media and exhibits a typical excimer emission peaked at 485 nm along with monomer emission peaked at 375 nm with a low detection limit (5 nM). The enhancement of both excimer and monomer emissions was accounted for the unique molecular architecture of sandwich conformation at ground sate prior the binding to LPS. The conformational search at ground state using ConfGen method with OPLS_2005 force field in Schrödinger software provided three distinct clusters of **BPTG** namely: sandwich-, semi- and open-bisguanylhydrazone pyrene. The sandwich form was appeared to be the lowest energy form in the DFT calculations using M06-2X functional in Gaussian 09 program. 2D NOESY spectrum provided evidence in a good agreement with both fluorescent and computational works. Importantly, **BPTG** is highly selective for LPS over heparin and other anionic biological species. Due to the expression of LPS on cell surface of Gram negative bacteria, **BPTG** was successfully applied as a fluorescent dye to visualize live *Vibrio cholerae*, life-threatening bacteria causing diarrhoeal disease.

**Keywords** : endotoxin sensing, lipopolysaccharide, fluorescent sensor, bisguanylhydrazone, pyrene

## 1.2 บทคัดย่อ

รหัสโครงการ : MRG5680030

ชื่อโครงการ : การพัฒนาฟลูออเรสเซนต์เซนเซอร์ เพื่อตรวจจับเอ็นโดท๊อกซิน (endotoxin) โดยใช้สารต้าน ภาวะเลือดเป็นพิษ (blood poisoning or sepsis) ชนิดใหม่

ชื่อนักวิจัย และสถาบัน : ดร.เกรียงศักดิ์ ขาวเนียม สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง

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### บทคัดย่อ

การติดเชื้อในกระแสเลือด (sepsis, blood poisoning) เป็นสาเหตุการเสียชีวิตที่สำคัญในกลุ่มผู้ป่วยที่มีภูมิต้านทานต่ำ ผู้ป่วยที่มีปัญหาโรคเบาหวานซึ่งมีแผลเรื้อรัง ผู้ป่วยที่ได้รับการผ่าตัดเปลี่ยนอวัยวะ และผู้ป่วยกลุ่มที่สูงอายุ หรือกลุ่มที่ต้องมีการฟอกไตเป็นประจำ ส่งผลให้อัตราการเสียชีวิตยังคงเพิ่มขึ้นอย่างต่อเนื่องจากทั่วทุกมุมโลก การติดเชื้อในกระแสเลือดเกิดจากปริมาณของสารห่อหุ้มลิโปโพลีแซคคาไรด์ของแบคทีเรียกลุ่ม Gram-negative ที่มีส่วนที่มีไขมันและน้ำตาลเป็นองค์ประกอบหลัก รวมทั้งมีหมู่ฟอสเฟตที่มีประจุลบอยู่สองหมู่ (lipopolysacharide, LPS) อยู่ในกระแสเลือด ส่งผลให้เกิดการตอบสนองของภูมิต้านทานของร่างกายอย่างรุนแรง ซึ่งถ้าการรักษาทำไม่ได้อย่างทันท่วงทีก็นำไปสู่การเสียชีวิตของผู้ป่วยได้ โครงการวิจัยนี้ได้ออกแบบการพัฒนาฟลูออเรสเซนต์เซนเซอร์เพื่อตรวจจับเอ็นโดท๊อกซิน (endotoxin) โดยใช้สารต้านภาวะเลือดเป็นพิษ (blood poisoning or sepsis) ชนิดใหม่ โดยมีโครงสร้างเป็น Bispyrenyl terephtalaldehyde-bis-guanylhydrazone (**BPTG**) เพื่อตรวจจับและวัดปริมาณ LPS ในตัวอย่าง ซึ่งสามารถให้ผลในการวัดได้อย่างรวดเร็วทันทีเมื่อมีการหยดสารฟลูออเรสเซนต์เซนเซอร์ลงในตัวอย่าง เราพบว่าฟลูออเรสเซนต์เซนเซอร์ที่พัฒนาขึ้นมาสามารถวัดปริมาณ LPS ได้ที่ความเข้มข้นต่ำถึง 5 นาโนโมลาร์ โดยมีการปล่อยแสง เอ็กซิเมอร์ที่ความยาวคลื่น 485 นาโนเมตร ควบคู่กับแสงโมเมอร์ที่ความยาวคลื่น 375 นาโนเมตร ซึ่งสามารถประยุกต์ใช้ได้กับการย้อมแบคทีเรียชนิดแกรมลบที่มีองค์ประกอบเป็น LPS อยู่ด้านนอกของผนังเซลล์ ในที่นี้ เราได้ย้อมแบคทีเรีย *Vibrio cholera* ซึ่งก่อให้เกิดโรคอหิวาตกโรคในขณะที่มีชีวิตได้อีกด้วย ดังนั้นการนำเอาองค์ความรู้และฟลูออเรสเซนต์เซนเซอร์ที่พัฒนาขึ้นมาต่อยอดเพื่อนำไปสู่การใช้จริงในการพัฒนาเป็นชุดตรวจสอบในทางคลินิกเป็นสิ่งที่ควรทำในอันดับถัดไป

**Keywords** : เอ็นโดท๊อกซิน, ลิโปโพลีแซคคาไรด์, ฟลูออเรสเซนต์เซนเซอร์, บิสกัวนิวไฮดราโซน, ไพรีน

## 2. Executive Summary

Despite tremendous advances in antimicrobial chemotherapy, the incidence and mortality rates due to the sepsis continue to escalate worldwide. The burden of disease is very stressful among patients with diabetics and organ transplant including elderly people. Sepsis risk factors of bacterial infections are more concerned with patients receiving chronic kidney dialysis. Lipopolysaccharide (LPS) acts as an endotoxin, and induces a strong response from the normal animal immune system. It contains common amphipathic constituent of the LPS of all Gram-negative bacteria and is composed of a hydrophilic, negatively charged *bis*-phosphorylated diglucosamine backbone, and a hydrophobic domain. A clinical treatment of septicemia needs a fast and effective endotoxin detection prior making medical decision to treat patient. We envision that *bis*-cationic nature of the terephthalaldehyde-*bis*-guanylhydrazone has potential use in endotoxin detection. Thus, we have developed novel bispyrene compound (**BPTG**) as a selective lipopolysaccharide (LPS) sensor. Probe **BPTG** exhibits high slectivity and sensitivity toward LPS as fluorescence 'off-on' behavior in HEPES-buffered DMSO-$H_2O$ (1:6(v/v), HEPES = 10 mM, pH = 7.4) with a low detectionlimit of 5 nM. The turn-on flurescence sensing of the LPS occurs through monomer and excimer emissions. The mechanism of probe was supported by computational experiment and found to be unique for its sandwich conformation, self-quenching form at ground state prior the binding to LPS with a butterfly-like skeleton. Upon binding with LPS in aqueous media, the probe shows a dose-dependent increase of fluorescent emissions and exhibits a typical excimer emission peaked at 485 nm along with monomer emission peaked at 375 nm. BPTG is highly selective for LPS over heparin and other anionic biological species. Due to the expression of LPS on cell surface of Gram negative bacteria, **BPTG** was successfully applied as a fluorescent dye to visualize live *Vibrio cholerae*, life-threatening bacteria causing diarrhoeal disease.

## 3. Background and Objective

### 3.1 Introduction

In Thailand, sepsis mortality rate has been continuously increasing from 11.1 in 100,000 population in 1995 to 44.7 in 100,000 population in 2011. Lipopolysaccharide (LPS) acts as an endotoxin, and induces a strong response from the normal animal immune system. Despite tremendous advances in antimicrobial chemotherapy, the incidence and mortality rates due to the sepsis continue to escalate.There are about 18 million people was affected by sepsis worldwide every year in which a mortality rate of nearly 30%. Even more sepsis kills 6,000,000 newborn and young children in developing countries each year. The burden of sepsis remains the challenge on health care system.

Sepsis is a severe illness caused by overwhelming infection of the bloodstream by outer membrane of Gram-negative bacteria which is composed of lipopolysaccharide (LPS).LPS acts as an endotoxin, and induces a strong response from the normal animal immune system. It is the uncontrolled and precipitous systemic inflammatory response that ultimately results in the fatal shock syndrome characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple organ failure.

Lipid A is a common amphipathic constituent of the LPS of all Gram-negative bacteria and is composed of a hydrophilic, negatively charged *bis*-phosphorylated diglucosamine backbone, and a hydrophobic domain. LPS is a potential drug target as well as a good biomarker since it plays a prominent role in raising the immune response that causes sepsis. The development of a more robust and more specific fluorescent probe is highly desirable for time-critical diagnosis and treatment of sepsis patients.

In 2010, an alkylated ammonium-pyrene probe was reported by Lee's group[1] for LPS detection at a low detection limit (100 nM) by supramolecular assembly. Pyrene is a very useful fluorescent probe for hydrophobicity.[2] Furthermore it can form intermolecular excimers in close proximity through $\pi$–$\pi$ interactions of stacking aromatic rings, in which the two aromatic rings are facing at a distance of ~3-4 Å.[3] The change in the surrounding environment of these multi-fluorophores could result in promoting or preventing the excimer formation. Hence, this excimer emission phenomenon has been used extensively in fluorescent chemosensors for nucleic sensing,[4,5,6] pH detection,[7] metal ion detection,[8] explosives detection[9] and fluoride ion sensing.[10]

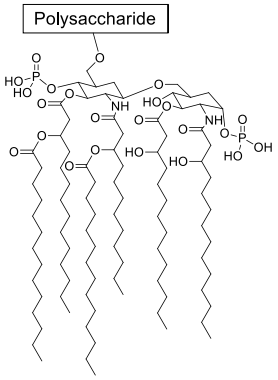



**Fig. 1** Molecular structure of lipopolysaccharide.[11]

We became interested in the excimer sensing approach since it has been reported that the formation of intramolecular excimers is independent of their concentrations[12] which should be an advantage for both medical application and as a tool for biological studies. This led us to consider the development of a LPS fluorescent

probe based on our earlier work on bis-cationic aminoguanidine for LPS sequestering.[13] The complementary length of distance between the two guanidinyl groups and the distance between the two phosphate groups in LPS (Fig. 1) provides a suitable electrostatic interaction, while the alkyl chains synergize the binding force with LPS through hydrophobic interactions. These two recognition elements seem to be very important to discriminate LPS from other phosphate species.

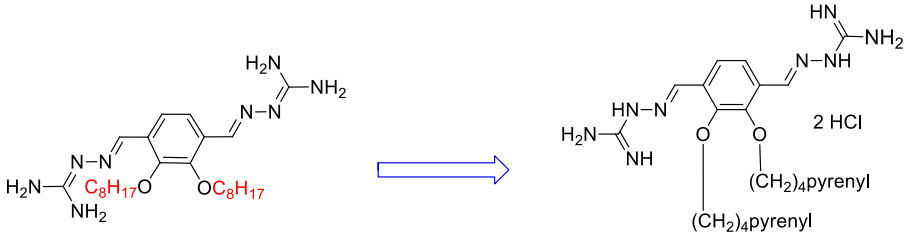
### 3.2 Objectives

The need for a better method to detect LPS in bloodstream or body fluids led us to consider the development of novel fluorescent probes from our earlier reported endotoxin sequestering agent. In fact, the charge-charge interactions of *bis*-phosphate groups on lipid A with *bis*-cationic portions of *bis*-guanylhydrazone scaffold was clearly demonstrated a strong binding mode comparable to known topical antibiotic agent (Polymixin B). Polymyxin B (PMB), a cationic amphiphilic cyclic decapeptide antibiotic isolated from *Bacillus polymyxa* has been known to bind lipid A and to neutralize of LPS. *Hence, the utilization of bis-phosphate moieties on lipid A as a docking site for designed fluorescent probes would allow a simple means with good selectivity to monitor LPS level in clinical samples.* Thus, the specific aims of this proposal are to:

1. Preparation of a novel fluorescent sensor that can function as excimer-monomer switch
2. Determination of the binding and selectivity of synthesized sensors against LPS

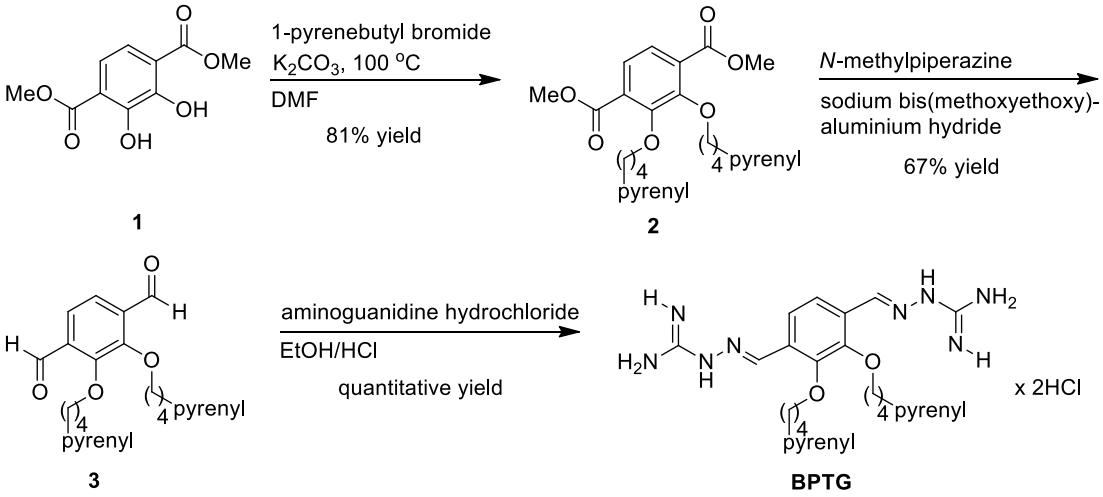
## 4. Research Methodology

Although there are many efforts to detect LPS, *bis*-cationic aminoguanidine molecule has never been explored before for fluorescent probe development. Among phosphate moieties in biological system, *bis*-phosphate of diglucosamine backbone embedded in LPS is the most unique one. We envision that *bis*-cationic nature of the terephthalaldehyde-*bis*-guanylhydrazone scaffold would be a good template to develop a series of small molecule sensors with effective selectivity for LPS detection through electrostatic interactions with *bis*-phosphate portion of lipid A. With the use of terephthalaldehyde-*bis*-guanylhydrazone scaffold as template, we proposed to replace both O-alkyl chains with O-alkyl-pyrene groups to produce *bis*-pyrene fluorescent probe as shown in Fig 2.




**Most potent compound for LPS binding** **Proposed compounds**

**Fig. 2** Proposed “excimer-monomer switching” fluorescent sensors




**Scheme 1.** Synthesis of the LPS sensor **BPTG**.

### 4.1 Reagents and Apparatus

Chemical reagents were used as purchased from Tokyo Chemical Industry Co., Ltd. (TCI), unless otherwise noted. All analytical chemicals were purchased from TCI Company without further purification. Lipopolysaccharide (LPS) was purchased from Sigma Company, which is obtained from Escherichia Coli 055:B5. All deuterated solvents were used as purchased from Cambridge Isotope Labs. Flash column chromatography was carried out using silica gel 60(230-400 mesh), while thin-layer chromatography (TLC) was carried out on silica gel HLF, precoated glass plates. All yields reported refer to isolated material judged to be homogeneous by TLC and NMR spectroscopy. The water was purified by Millipore filtration system.

### 4.2 Analytical Instrument

Nuclear magnetic resonance (NMR) spectra were recorded in methanol-$d_4$ or deuterated chloroform ($CDCl_3$) on a Bruker 400 MHz spectrometer. UV-vis absorption spectra were recorded a Varian Cary 50 UV-Vis spectrophotometer (Varian, USA) at 25 °C. Fluorescence spectroscopy was performed on an EnVision® Multilabel Reader (PerkinElmer Inc.). High-resolution electrospray ionization mass spectrometry was performed on a Bruker maxis using $CH_3CN$ as a solvent.

### 4.3 Synthesis of Compound 2

A solution of bromopyrene (100 mg, 0.44 mmol) in DMF 2mL was added slowly to a stirred mixture of dimethyl 2,3-dihydroxyterephthalate (297 mg, 0.88 mmol) and $K_2CO_3$ (150 mg, crushed to a powder) in DMF (3mL). The resulting mixture was heated and stirred overnight at reflux. All the volatiles were removed *in vacuo*, and the residue was partitioned between water and DCM. The organic layer was washed with brine and dried over $MgSO_4$. The crude product was purified by silica- column chromatography (Hexane:EtOAc = 4:1) to give the desired product as pale yellow liquid 263 mg (yield 81 %). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.9 (8H, m), 3.2 (4H, t, $J$ = 7.3 Hz), 3.8 (6H, s), 4.1 (4H, t, $J$ = 6.2 Hz), 7.5 (2H, s), 7.7-8.1 (18H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 28.0, 30.1, 33.0, 52.2, 74.8, 123.2, 124.5, 124.6, 124.7, 124.9, 125.1, 125.6, 126.4, 127.0, 127.3, 128.5, 129.7,

129.7, 130.7, 131.3, 136.4, 153.1, 165.9 ppm; IR (neat, NaCl): 3040, 2924, 2854, 1727, 1602, 1434, 1283, 1260 1037, 843, 757 $cm^{-1}$; ESI-HRMS: m/z calc'd for $[C_{50}H_{42}NaO_6]^+$, 761.2874 found 761.2876.

**4.4 Synthesis of Compound 3**

A solution of N-methylpiperazine in toluene (0.90 g, 9.02 mmol, 1 mL) was added to a solution of sodium bis(methoxyethoxy) aluminum hydride (70% toluene solution, 1.04 g, 5.12 mmol, 2 mL) at 0 °C for 0.5 h. The obtained toluene solution was added to a solution of 2 (100 mg, 0.14 mmol) in toluene (0.5 mL) at -20 °C over 50 min, and the mixture was stirred at room temperature for 30 min. The reaction was quenched by adding water (20 mL) and toluene (5 mL). The insoluble materials were filtered off and the filtrate was washed with 1 N HCl solution, water, and brine. The organic layer was dried over anhydrous $Na_2SO_4$, and then concentrated *in vacuo*. Flash chromatography ($CH_2Cl_2$) of the residue gave 3 as a yellow liquid (61.6 mg Yield: 67%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 11.9 (8H, m), 3.3 (4H, t, *J* = 7.2 Hz), 4.1 (2H, t, *J* = 6.2 Hz), 7.6 (2H, s), 7.7-8.2 (18H), 10.4 (2H, s) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 28.0, 28.9, 33.0, 75.7, 122.7, 123.1, 124.7, 124.8, 124.9, 125.1, 125.8, 126.7, 127.1, 127.3, 127.4, 129.9, 130.8, 131.4, 134.4, 135.9, 156.0, 189.3 ppm; IR (neat, NaCl): 3526, 3039, 2937, 2864, 1686, 1602, 1439, 1376, 1243, 1161, 1036, 842, 758 $cm^{-1}$; ESI-HRMS: m/z calc'd for $[C_{48}H_{38}O_4{}^+2H_2O]^+$, 714.2976 found 714.0608 and $[C_{48}H_{38}O_4{}^+H_2O]^+$, 696.2870 found 696.3110.

**4.5 Synthesis of BPTG**

To a hot solution at 70 °C of aminoguanidine hydrochloride (33.17 mg, 0.3 mmol) in EtOH (1 mL) was added a solution of 3 (100 mg, 0.15 mmol, in 0.5 mL EtOH) and conc. HCl (0.1 mL). The reaction mixture was heated up to 80 °C for 1 h. The reaction mixture was cooled to 0 °C. The resultant precipitate was filtered and washed with cold ether (2 mL) to give **BPTG** as a white solid 127 mg (quantitative yield). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.7 (8H, m), 3.0 (4H, t, *J* = 6.7 Hz), 3.9 (4H, t, *J* = 6.1 Hz), 7.4 (2H, s), 7.5-8.0 (18H), 8.22 (s, 2H) ppm; $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta$ 28.1, 30.1, 33.1, 74.9, 123.3, 124.6, 124.7, 125.8, 125.7, 126.5, 127.1, 127.4, 128.5, 129.8, 130.9, 131.4, 136.5, 152.7, 166.2 ppm; IR (neat, NaCl): 3523, 2925, 2854, 1739, 1674, 1629, 1457, 1379, 1245 $cm^{-1}$; ESI-HRMS: m/z calc'd for $[C_{50}H_{47}N_8O_2]^+$, 791.3816 found 791.3816.

**4.6 Study of Photophysical Properties**

A stock solution of **BPTG** (10 mM) in a mixed solution of pure DMSO and 10 mM HEPES buffer pH 7.4 (1 : 6 v/v) was prepared. The UV-vis absorption spectra were recorded in 1 cm quartz cells in the wavelength range from 225 nm to 600 nm at room temperature. The fluorescence spectra were recorded from 365 nm to 665 nm at room temperature using an excitation wavelength of 342 nm. The fluorescence quantum yield of **BPTG** was calculated from absorbance (below 0.05) by comparing it to standard quinine sulfate ($\Phi$F = 0.54) in 0.1 M $H_2SO_4$. The standard curve was prepared from dilutions of the stock solution of LPS ranging from 5 nM to 50 $\mu$M. Then, the optimized conditions for the LPS sensor were investigated.

**4.7 Fluorescence emission Measurements**

A stock solution of **BPTG** (100 mM) in pure DMSO was prepared. A stock solution of 1 mM LPS was prepared by dissolving in 10 mM HEPES buffer pH 7.4. A mixed solution of BPTG (100 mM, 0.5 mL) and LPS

solution (1 mM, 50 mL) at room temperature was adjusted to 5.0 mL by adding the mixture of DMSO/10 mM HEPES buffer pH 7.4 (1 : 6 v/v) to afford final concentrations of 100 μM BPTG and 100 μM LPS. At a time-point 5 minutes after mixing, the fluorescence emission spectrum was recorded from 365 nm to 665 nm using an excitation wavelength of 342 nm. An interference study using other biological anionic species with this method utilized a stock solution of 10 mM of other common acid and phosphate species (malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate, lecithin, and heparin) in 10 mM HEPES buffer pH 7.4. A mixture of BPTG (50 μM), LPS (5 μM) and each of these interfering species (50 μM) were prepared. The fluorescence emission spectra were recorded at the single wavelength of 485 nm, using an excitation wavelength of 342 nm. All measurements were taken after 5 minutes of mixing at room temperature.

### 4.8 Computational Experiment

Different conformations of the bispyrenyl bis-guanylhydrazone were obtained from the conformational search in Schrödinger software by using ConfGen method with OPLS_2005 force field. The maximum number of examined conformations was set to 1000. The DFT calculations with M06-2X functional provided the stable local minima of the BPTG clusters; namely: sandwich-, semi- and open-stacking based on the folding of pyrene to bis-guanylhydrazone. All structures were optimized with Gaussian 09 program. The 6-31G(d,p) basis set was used for all atoms. The molecular orbital and charges were calculated with Natural Bond Orbital (NBO) analysis.

## 5. Results and Discussion

A novel fluorescent probe to detect LPS based on bispyrenyl terephtalaldehyde-bis-guanylhydrazone (**BPTG**) was prepared by a straighforward three-step sequence in high yield. 2,3-Dihydroxyterephthalic acid dimethyl ester was alkylated with 1-pyrenebutyl bromide using $K_2CO_3$ as the base in DMF to give **2** in 81% (Scheme 1). The aldehyde **3** was obtained in 67% yield by partial reduction of **2** with sodium bis(methoxyethoxy)-aluminium hydride (Red-Al) in the presence of N-methylpiperazine.[14] The **BPTG** was obtained from the condensation of terephtaldehyde **3** and the hydrochloride salt of aminoguanidine in refluxing ethanol and hydrochloric acid. Pure **BPTG** was isolated as a crystalline hydrochloride salt after cooling to 0 °C and washing with cold ether.

### 5.1 LPS Sensing

To our surprise, upon the addition of LPS into the **BPTG** solution, green excimer fluorescence (peaked at 485 nm) was observed with a characteristic feature of excimer emission along with an increase of monomer emission around 375 nm (Fig. 2). The enhancement of both excimer and monomer emissions of the bispyrenyl system is not usual since most sensors based on the two pyrene units linked covalently with central molecule show ratiometric fluorescence response with the changes of excimer-monomer emissions which involved the reduction of monomer band and the increase of excimer emission upon the binding to targets and vice versa. Although bispyrenyl molecule tends to form excimer in aqueous medium due to the lipid nature of pyrene, it is known that

aromatic compounds can intercalate to quench the excimer-monomer emissions. So, the molecular orientation of **BPTG** could play an important role in fluorescent quenching process prior the addition of LPS.

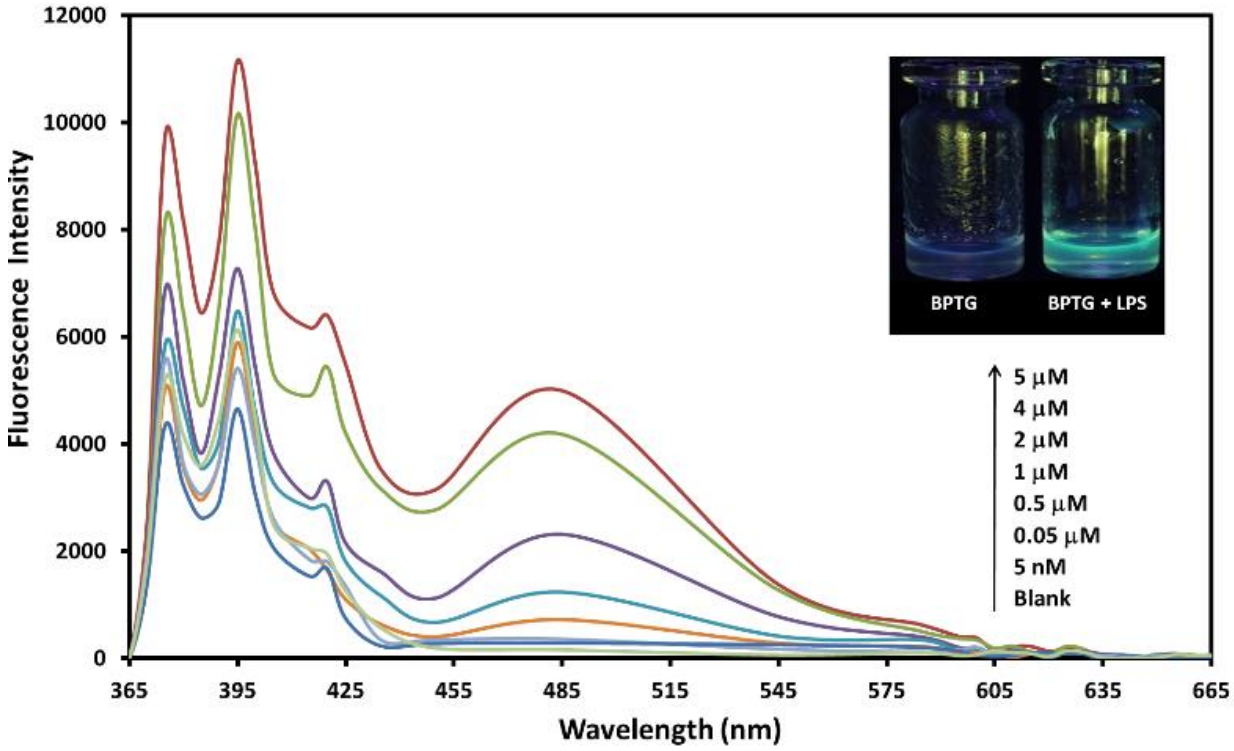



**Fig. 3** Changes in fluorescence emission spectra of **BPTG** (10 μM) upon addition of LPS in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4). Excitation wavelength: 342 nm. (Inset) photograph of **BPTG** (10 μM) in the presence of 5 μM LPS in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4).

**5.2 Computational Analysis**

In order to better understanding the structural behavior of **BPTG**, a conformational search was done to obtain the low-lying stable structures at ground state of **BPTG** by using ConfGen39 method with OPLS_2005 force field in Schrödinger software. Three distinct conformations of **BPTG** as shown in Fig. 4; namely: sandwich-, semi- and open-stacking based on the folding of pyrene to bis-guanylhydrazone were later optimized by the DFT calculations with M06-2X functional in Gaussian 09 program to provide the stable local minima. The relative energies are 0.0, 4.4 and 15.5 kcal $mol^{-1}$ for sandwich-, semi- and open-stacking conformations, respectively.

A parallel and co-facial alignment of sandwich stacking with a highly congruent HOMO and LUMO in Fig. 4 suggests that a pairing form between pyrenes and bis-guanylhydrazone prevents the formation of a pyrene excimer in aqueous buffer via a special π stacking with a butterfly-like skeleton. The distances between each pyrenyl units and the phenyl ring of the terephtalaldehyde-bis-guanylhydrazone are in the range of 3-4 Å. The HOMO density, the π-electron donor, is mainly located on one pyrenyl ring; meanwhile the LUMO density, the π-electron acceptor, is mainly located on the bis-guanylhydrazone system in the center part of the sandwich stacking. These molecular characters could ultimately play an important role in fluorescence quenching of monomer and excimer emission through photoinduced electron transfer (PET) prior to the binding with LPS.

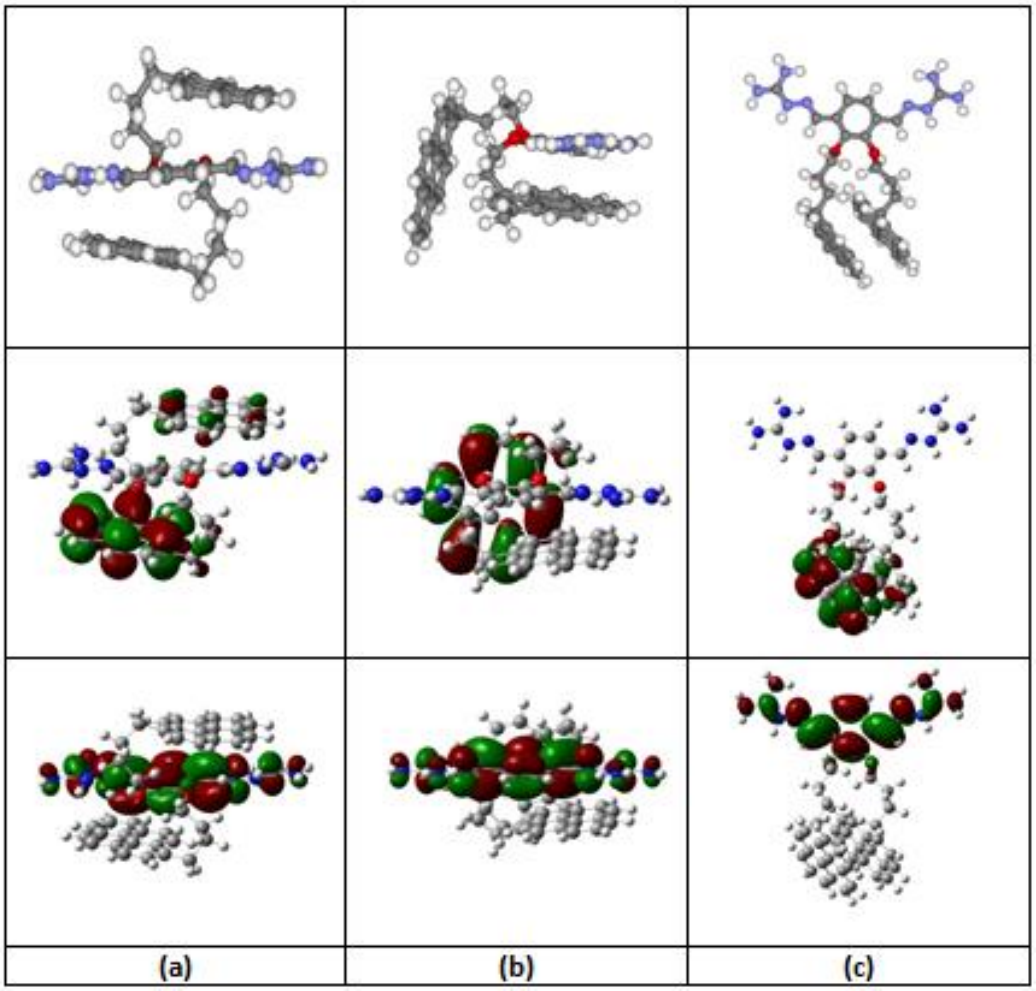
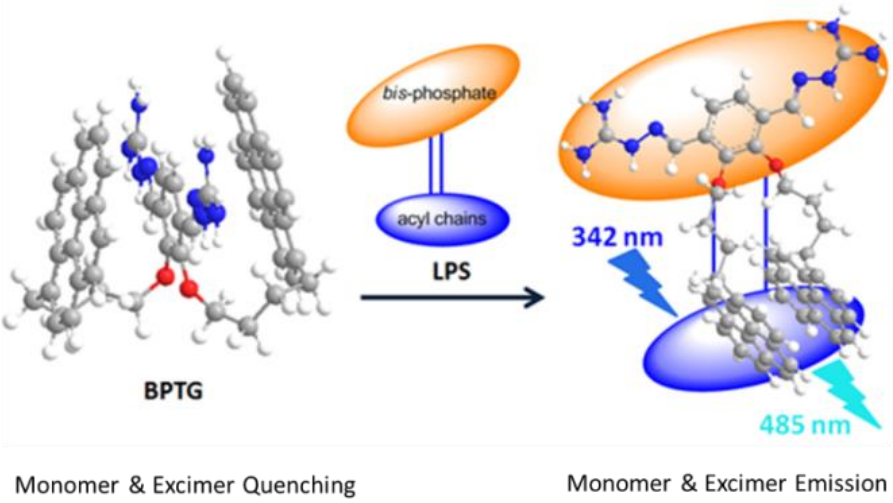



**Fig. 4** Conformations of **BPTG**, HOMO (second row) and LUMO (third row) of (a) sandwich- (b) semi- and (c) open-stacking conformations optimized with the M06-2X functional in Gaussian 09 program.

On the basis of theoretical studies, the plausible mechanism of **BPTG** in the present system is schematically depicted in Scheme 2 accounting for the excimer-monomer emission 'turn-on' in aqueous solution. Upon the binding event of LPS and **BPTG**, the aminoguanidine groups form salt bridges with phosphate groups of LPS, meanwhile pyrene units of **BPTG** interact with acyl chains of LPS via the lipophilic interactions. Therefore, it is most likely that pyrenyl units are loosen off from the sandwich stacking and drawn into close proximity to form a pair of excimer with monomer and excimer emissions.




**Scheme 2.** Proposed mechanism of the ‘‘turn-on mode’’ of **BPTG** after addition of LPS.

### 5.3 Relative Fluorescent Signal ($I/I_0$) of BPTG

Upon the addition of LPS (5 μM), the *I/I0* of **BPTG** (10 μM) exhibited the highest ratio about 30 times as shown in Fig.5 and the quantum efficiency increased to 25% due to the conformation change allowing excimer formation. The detection limit of **BPTG** for LPS detection was measured by using the relative fluorescent signal plot *(I/I0)* against LPS concentrations ranging from 0 to 5 μM, and this plot provided a good linear correlation (Fig. 5). The limit of detection determined by the plot was 5 nM under our experimental conditions.

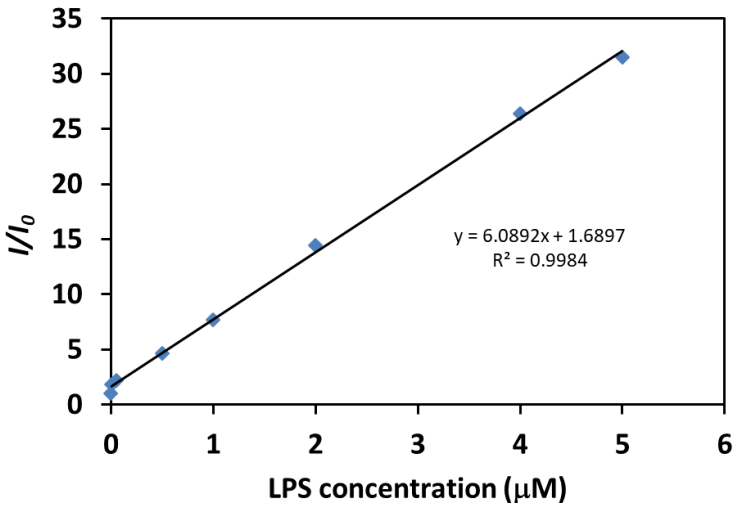



**Fig. 5** Relative fluorescent signal (*I/I0*) of **BPTG** ($I_{485}$ upon addition of LPS 0-5 μM, $\lambda_{ex}$ = 342 nm) in a solution of DMSO/HEPES buffer (10 mM, pH 7.4).

### 5.4 Job’s Plot

In order to understand the effect of the stoichiometric ratio of BPTG to LPS, we carried out a Job’s plot analysis and the result suggested that the stoichiometric ratio of BPTG to LPS was 4:1 (Fig. 6).

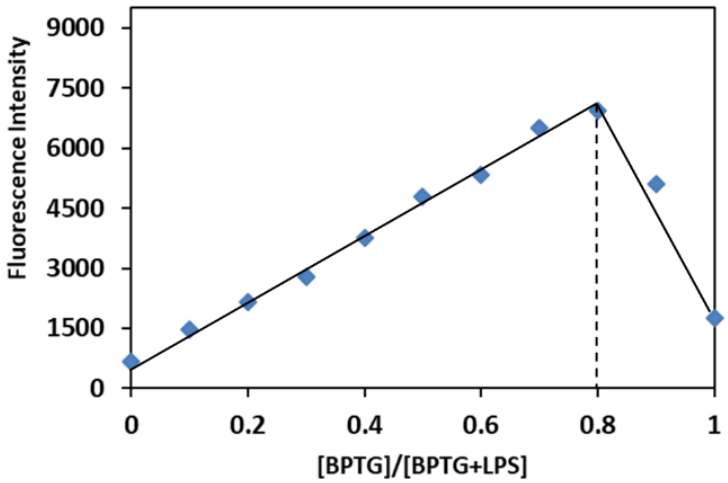



**Fig. 6** Job's plot of the relationship between fluorescence intensity of **BPTG** (100 μM) in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4) and mole fraction of LPS ($\lambda_{em}$ = 485 nm).

### 5.5 Selectivity of BPTG

To better investigate the practical applicability of **BPTG** as LPS fluorescence sensor, competitive experiments were carried out in the presence of LPS mixed with malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate, lecithin, and heparin as shown in Fig. 7. No significant interference in the detection of LPS with the sensor **BPTG** was observed in the presence of most competitive acid and phosphate biological species. Accordingly, these observations suggested that the sensor **BPTG** can be used as selective fluorescent sensor for LPS in the presence of most biological anionic species.

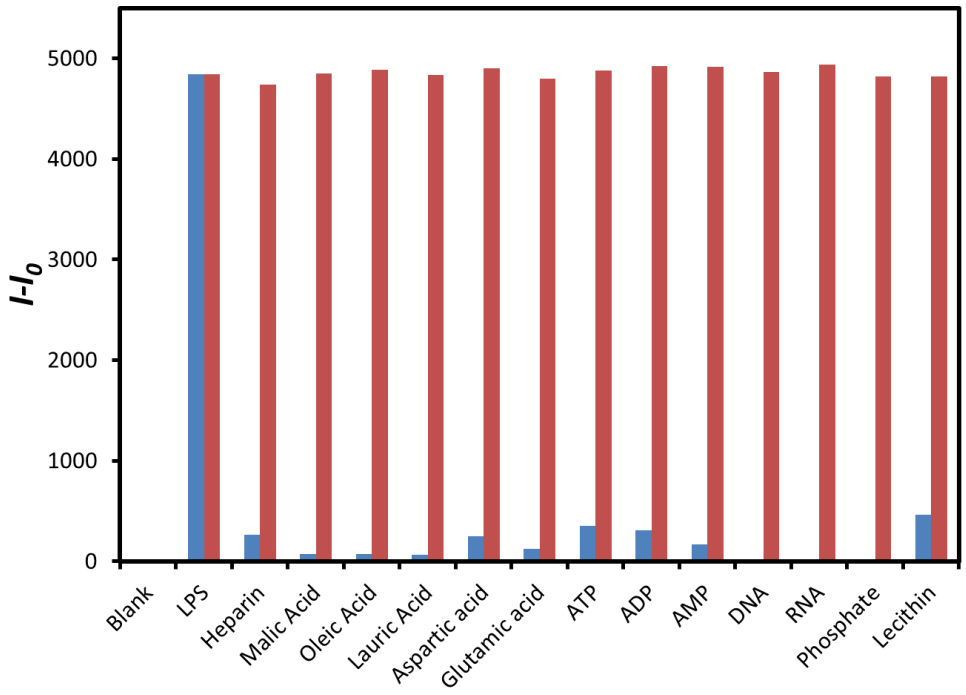



**Fig. 7** Selectivity of **BPTG** (10 μM) to some biologically important anionic species (50 μM) in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4). heparin, malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate and lecithin. Blank refers to free **BPTG** (10 μM) in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4).

Heparin is regarded as one of the clinical important medicines for preventing blood clot during heart surgery or treatment of certain bleeding disorder. **BPTG** showed a slight change of fluorescence at 485 nm at high concentration (50 μM) due to the lack of the alkyl chains. To the best of our knowledge, **BPTG** is the first chemosensor among reported small molecule probes that can sense LPS over heparin. A small enhancement of fluorescence emission at 485 nm was observed with lecithin since it possesses long alkyl chains and the negatively charged phosphate that can interact with aminoguanidine moieties of **BPTG**.

### 5.6 Visualization of *V.cholerae*

To demonstrate the application of **BPTG** as a tool for biological study, *Vibrio cholerae*, Gram-negative and virulent bacteria to cause diarrhoeal disease, was incubated with **BPTG**. Green fluorescence was observed with a clear black background to reveal living bacteria colonies due to the expression of LPS on cell surface as shown in Fig. 8.

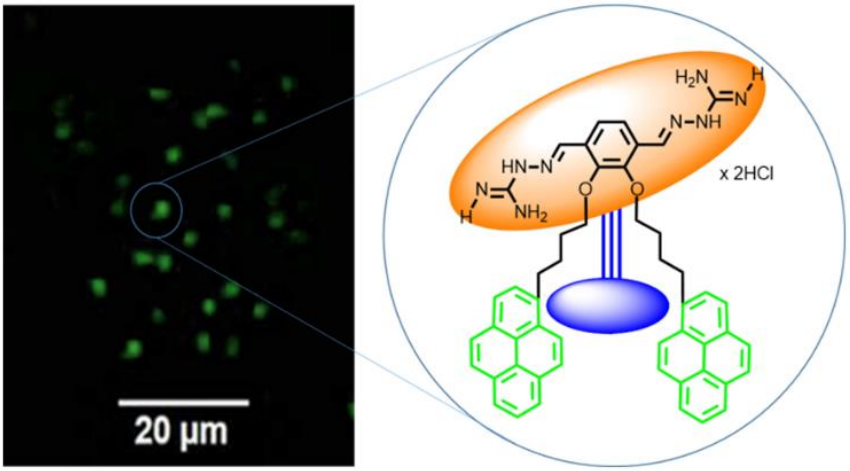



**Fig. 8** Visualization of *V.cholerae* (~$10^6$ cells/mL) after incubation with **BPTG** (10 μM). Cells were imaged under Olympus FV10i at 100x magnification.

## 6. Conclusions and Suggestions

In summary, the development of **BPTG** for LPS sensing led us to discover the unique molecular feature of **BPTG** as a self-quenching molecule prior to binding to LPS. **BPTG** is a turn-on probe for LPS detection and displays monomer and excimer emissions in aqueous solution with the lowest of detection limit at 5 nM. **BPTG** is highly selective for LPS over heparin and other anionic biological species. The unique molecular architecture of **BPTG** provides a better understanding for the rational design of fluorescence sensors. Furthermore, the molecular design of **BPTG** as a turn-on sensor could be used for fluorescent-sensor developments of other target detections as well.

## 7. Output (Acknowledge the Thailand Research Fund)

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### 7.2 International Journal Publication

**Kriangsak Khownium**,* Jariya Romsaiyud, Suparerk Borwornpinyo, Preedajit Wongkrasant, Pawin Pongkorpsakol, Chatchai Muanprasat, Bundet Boekfa, Tirayut Vilaivan, Somsak Ruchirawat, and Jumras Limtrakul; **Turn-on Fluorescent Sensor for the Detection of Lipopolysaccharides Based on a Novel Bispyrenyl Terephtalaldehyde-Bis-Guanylhydrazone**, *New Journal of Chemistry*, (2019), 43, 7051-56. (Impact Factor 3.07)

# 8. Appendix

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**Name**

Dr. Kriangsak Khownium

**Academic position**

Lecturer

**Education**

**2003-2009: Ph.D.** Department of Medicinal Chemistry, University of Kansas, (KS), USA;

Advisor: Professor Gunda I. Georg

**2000-2003: M.S.** Department of Chemistry, Oregon State University, (OR), USA;

Advisor: Professor Kevin P. Gable

**1996-2000: B.S.** Chemistry Department, Kasetsart University, Bangkok, Thailand;

Advisor: Professor Jumras Limtrakul, First Class Honors in Chemistry

**Work / Research Experiences**:

**March 2016-present: Lecturer** in Faculty of Medicine, King Mongkut's Institute of Technology Ladkrabang, Bangkok

*Research projects:* Drug design using computational method and Medical innovation using IoT and AI

**March 2015-February 2016: Senior Researcher** in Medicinal Chemistry, Excellent Center for Drug Discovery, Faculty of Science, Mahidol University, Bangkok

*Research projects:* 1. Development of lipopolysaccharide removal materials for application in medical cartridge

2. Drug design using computational methods and structure activity relationship analysis in the development of antifolates against resistance-compromised targets

**June 2012-February 2015: Lecturer** in Organic Chemistry Division, Chemistry Department, King Mongkut's University of Technology Thonburi, Bangkok

*Research projects:* 1. Probe development for LPS detection (completed in June 2015)

2. Syntheses and structure activity studies of rhinacanthone derivatives

3. *In silico* screening and the molecular library design for MTH1 inhibitors as anticancer agents

**August 2010- May 2012: Researcher** in Laboratory of Medicinal Chemistry under the supervision of Professor Dr. Somsak Ruchirawat, Chulabhorn Research Institute**,** Bangkok

*Research project:* Development of lamellarin phosphate prodrugs for water soluble anticancer agents

**2004-2009: Ph.D. Research,** 2004-2007: Department of Medicinal Chemistry, University of Kansas, Kansas, and 2007-2009: Department of Medicinal Chemistry, University of Minnesota, Minnesota, USA
Advisor: Professor Gunda I. Georg
*Ph.D. Thesis:* Synthesis and evaluation of modified oximidine analogues as anticancer agents and of terephthalaldehyde-*bis*-guanylhydrazones as endotoxin sequestering agents.

**2001-2003: M.S. Research,** Department of Chemistry, Oregon State University, Oregon, USA
Advisor: Professor Kevin P. Gable

*M.S. Thesis: Direct Atom Transfer vs. Ring Expansion in Reaction of Rhenium Oxo Complexes with Cyclooctene Epoxides and Episulfides.*

**1999-2000: B.S. Research,** Chemistry Department, Kasetsart University, Bangkok, Thailand
Advisor: Professor Jumras Limtrakul
*B.S. Research Project: Synthesis and Characterization of Ti-containing Silicate Zeolite having MFI structure*.

**Expertise:**

- Medicinal chemistry strategies (SAR profiling, lead optimization, computational analysis)
- Drug design using computational method
- Multi-step organic synthesis
- Modern analytical and purification methods: NMR (1, 2D), FT-IR, HPLC, LC/MS, MS
- Combinatorial chemistry/parallel synthesis, SPE techniques, microwave-assisted synthesis
- Functional Food Formulation
- Medical Innovation

**List of Special Topic-Classes Attended:**

- **Process Chemistry Workshop**, Syrris Ltd, Sithiporn Associates CO., LTD. Bangkok, Instructor Omar Jina, Global Head of Sales and Support, May 9-10, 2011
- **Drug Design Class**, Department of Medicinal Chemistry, University of Kansas, Lawrence, Instructor Lester A. Mitscher, University Distinguished Professor of Medicinal Chemistry, December 19-20, 2005
- **IRORI Solid Phase Organic Chemistry, Combinatorial Chemistry Workshop**, Department of Medicinal Chemistry, University of Kansas, instructor Thomas Beattie, Lawrence May 5-8, 2004

**Awards and Affiliations:**

**2018:** Gold Medal Award with Congratulations of Jury for **Smart Road Surface Monitoring System via Cloud Computing** from 46$^{th}$ International Exhibition of Inventions Geneva, Switzerland

**2017:** Gold Medal Award for **A Time-Release Formulation of Medicinal Plant Extracts for Diabetic Adjunctive Treatment** from 45$^{th}$ International Exhibition of Inventions Geneva, Switzerland

**2010:** Medicinal Chemistry Travel Grant Award from ACS Division of Medicinal Chemistry

**1993-2006:** Scholarship for the Development and Promotion of Science and Technology Talents (DPST) from the Royal Thai Government

**1999:** Outstanding Academic Performance Award from the Siam Cement Public Company Limited, Thailand

**1997-1999:** Outstanding Academic Performance Award from Faculty of Science, Kasetsart University, Thailand

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1. Kriangsak Khownium, Jariya Romsaiyud, Suparerk Borwornpinyo, Preedajit Wongkrasant, Pawin Pongkorpsakol, Chatchai Muanprasat, Bundet Boekfa, Tirayut Vilaivan, Somsak Ruchirawat, and Jumras Limtrakul; **Turn-on Fluorescent Sensor for the Detection of Lipopolysaccharides Based on a Novel Bispyrenyl Terephtalaldehyde-Bis-Guanylhydrazone**, *New Journal of Chemistry*, (2019), *43*, 7051-56. (Impact Factor 3.288)

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**Examples of recent research outputs:**

1. Teeranan Nongnual, Somkiat Nokbin, Pipat Khongpracha, Philippe Anthony Bopp, **Jumras Limtrakul** Density functional theory evidence for an electron hopping process in single-walled carbon nanotube-mediated redox reactions Carbon, **2010**, *48*, 1524 Impact Factor of 6.008

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Functional Theory (M06-2X) in ONIOM Scheme Langmuir, **2009**, *25*, 12990 Impact Factor of 4.186

### 8.3 Reprint and Supporting Information

NJC

PAPER



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# Turn-on fluorescent sensor for the detection of lipopolysaccharides based on a novel bispyrenyl terephtalaldehyde-bis-guanylhydrazone†

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A novel bispyrene compound (**BPTG**) was developed as a selective lipopolysaccharide (LPS) sensor. The **BPTG** probe exhibited high selectivity and sensitivity toward LPS with a fluorescence 'off–on' behavior in HEPES-buffered DMSO–$H_2O$ (1 : 6 (v/v), HEPES = 10 mM, pH = 7.4) with a low detection limit of 5 nM. The turn-on flurescence sensing of the LPS occurred through monomer and excimer emissions. The mechanism of the probe was supported by computational experiments and was found to be unique for its sandwich conformation and self-quenching ability at ground state prior to the binding to the LPS with a butterfly-like skeleton. Upon binding with LPS in an aqueous medium, the probe showed a dose-dependent increase in fluorescent emissions and exhibited a typical excimer emission peak at 485 nm along with a monomer emission peak at 375 nm. **BPTG** is highly selective for LPS over heparin and other anionic biological species. Due to the expression of LPS on the cell surface of Gram negative bacteria, **BPTG** was successfully applied as a fluorescent dye to visualize live *Vibrio cholerae*, which are life-threatening bacteria causing diarrhoeal disease.

## Introduction

Sepsis (also called blood poisoning or septicemia) is a major public health threat caused by a lipopolysaccharide (LPS), which is a component of the bacterial cell wall.[1,2] Sepsis is the number one cause of death in the intensive care unit, with a current estimate of at least 750 000 cases per year, and 215 000 fatalities in the United States annually.[3] Every year, about 18 million people are affected by sepsis worldwide with a mortality rate of nearly 30%.[4] LPS has been proposed as a potential drug target as well as a good biomarker since it plays a prominent role in initiating the immune response that causes sepsis.[5–12] Thus, the development of a robust and specific method is highly desirable for the time-critical diagnosis and treatment of sepsis patients.

In 2010, an alkylated ammonium–pyrene probe was reported by Lee's group[8] for LPS detection at a low detection limit (100 nM) by supramolecular assembly. Pyrene is a very useful fluorescent probe for hydrophobicity.[13] Furthermore, it can form intermolecular excimers in close proximity through π–π stacking interactions of aromatic rings, in which the two aromatic rings are facing at a distance of ~3–4 Å.[14,15] Changes in the surrounding environment of these multi-fluorophores could result in promoting or preventing the excimer formation. Hence, this excimer emission phenomenon has been used extensively in fluorescent chemosensors for nucleic acid sensing,[16–18] pH detection,[19] metal ion detection,[20–24] explosive detection[25,26] and fluoride ion sensing.[27]

We became interested in the excimer sensing approach since it has been reported that the formation of intramolecular excimers is independent of their concentrations,[29] which should be an advantage for both medical applications and as a tool for biological studies. This led us to consider the development of a

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9nj00323a

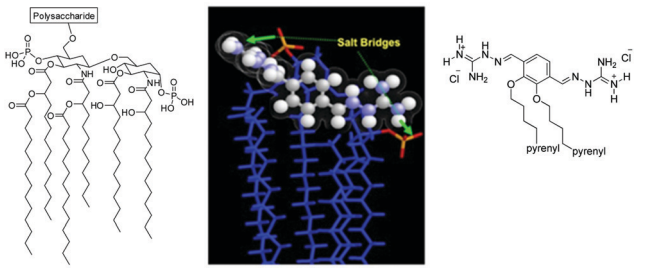



Fig. 1 The molecular structure of a lipopolysaccharide[28] and molecular binding of bis-cationic aminoguanidine species with LPS.

LPS fluorescent probe based on our earlier study on bis-cationic aminoguanidines for LPS sequestering.[5] The complementary distances between the two aminoguanidinyl groups and the two phosphate groups in LPS (Fig. 1) provide suitable electrostatic interactions, while the alkyl chains bearing pyrenyl groups synergize the binding force with LPS through hydrophobic interactions. These two recognition elements seem to be very important to discriminate LPS from other phosphate species.

# Experimental

### Reagents and apparatus

Chemical reagents were used as purchased from Tokyo Chemical Industry Co., Ltd (TCI), unless otherwise noted. All analytical chemicals were purchased from TCI and used without further purification. Lipopolysaccharide (LPS) was purchased from Sigma Company, which was obtained from *Escherichia coli* 055:B5. All deuterated solvents were used as purchased from Cambridge Isotope Labs. Flash column chromatography was performed using silica gel 60 (230–400 mesh), while thin-layer chromatography (TLC) was performed on silica gel HLF pre-coated glass plates. All yields reported refer to isolated materials judged to be homogeneous by TLC and NMR spectroscopy. The water was purified using a Millipore filtration system.

### Analytical instruments

Nuclear magnetic resonance (NMR) spectra were recorded in methanol-$d_4$ or deuterated chloroform ($CDCl_3$) on a Bruker 400 MHz spectrometer. UV-vis absorption spectra were recorded on a Varian Cary 50 UV-vis spectrophotometer (Varian, USA) at 25 °C. Fluorescence spectroscopy was performed on an EnVision® Multilabel Reader (PerkinElmer Inc.). High-resolution electrospray ionization mass spectrometry was performed on a Bruker maXis using $CH_3CN$ as a solvent.

### Synthesis of compound 2

A solution of bromopyrene (100 mg, 0.44 mmol) in DMF (2 mL) was added slowly to a stirred mixture of dimethyl 2,3-dihydroxyterephthalate (297 mg, 0.88 mmol) and $K_2CO_3$ (150 mg, crushed to a powder) in DMF (3 mL). The resulting mixture was heated and stirred overnight at reflux. All the volatiles were removed *in vacuo*, and the residue was partitioned between water and DCM. The organic layer was washed with brine and dried over $MgSO_4$. The crude product was purified using silica-column chromatography (hexane : EtOAc = 4 : 1) to give the desired product as a pale yellow liquid (263 mg, yield 81%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 1.9 (8H, m), 3.2 (4H, t, $J$ = 7.3 Hz), 3.8 (6H, s), 4.1 (4H, t, $J$ = 6.2 Hz), 7.5 (2H, s), 7.7–8.1 (18H) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 28.0, 30.1, 33.0, 52.2, 74.8, 123.2, 124.5, 124.6, 124.7, 124.9, 125.1, 125.6, 126.4, 127.0, 127.3, 128.5, 129.7, 129.7, 130.7, 131.3, 136.4, 153.1, 165.9 ppm; IR (neat, NaCl): 3040, 2924, 2854, 1727, 1602, 1434, 1283, 1260 1037, 843, 757 cm$^{-1}$; ESI-HRMS: $m/z$ calculated for $[C_{50}H_{42}NaO_6]^+$, 761.2874 found 761.2876.

### Synthesis of compound 3

A solution of *N*-methylpiperazine in toluene (0.90 g, 9.02 mmol, 1 mL) was added to a solution of sodium bis(methoxyethoxy) aluminum hydride (70% toluene solution, 1.04 g, 5.12 mmol, 2 mL) at 0 °C for 0.5 h. The obtained toluene solution was added to a solution of **2** (100 mg, 0.14 mmol) in toluene (0.5 mL) at −20 °C over 50 min, and the mixture was stirred at room temperature for 30 min. The reaction was quenched by adding water (20 mL) and toluene (5 mL). The insoluble materials were filtered off and the filtrate was washed with a 1 N HCl solution, water, and brine. The organic layer was dried over anhydrous $Na_2SO_4$, and then concentrated *in vacuo*. Flash chromatography ($CH_2Cl_2$) of the residue gave **3** as a yellow liquid (61.6 mg yield: 67%). $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 11.9 (8H, m), 3.3 (4H, t, $J$ = 7.2 Hz), 4.1 (2H, t, $J$ = 6.2 Hz), 7.6 (2H, s), 7.7–8.2 (18H), 10.4 (2H, s) ppm; $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 28.0, 28.9, 33.0, 75.7, 122.7, 123.1, 124.7, 124.8, 124.9, 125.1, 125.8, 126.7, 127.1, 127.3, 127.4, 129.9, 130.8, 131.4, 134.4, 135.9, 156.0, 189.3 ppm; IR (neat, NaCl): 3526, 3039, 2937, 2864, 1686, 1602, 1439, 1376, 1243, 1161, 1036, 842, 758 cm$^{-1}$; ESI-HRMS: $m/z$ calculated for $[C_{48}H_{38}O_4 + 2H_2O]^+$, 714.2976 found 714.0608 and $[C_{48}H_{38}O_4 + H_2O]^+$, 696.2870 found 696.3110.

### Synthesis of BPTG

To a hot solution at 70 °C of aminoguanidine hydrochloride (33.17 mg, 0.3 mmol) in EtOH (1 mL) was added a solution of **3** (100 mg, 0.15 mmol, in 0.5 mL EtOH) and conc. HCl (0.1 mL). The reaction mixture was heated up to 80 °C for 1 h. The reaction mixture was cooled to 0 °C. The resultant precipitate was filtered and washed with cold ether (2 mL) to give the bis-guanylhydrazone analogue **BPTG** as a white solid 127 mg (quantitative yield). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 1.7 (8H, m), 3.0 (4H, t, $J$ = 6.7 Hz), 3.9 (4H, t, $J$ = 6.1 Hz), 7.4 (2H, s), 7.5–8.0 (18H), 8.22 (s, 2H) ppm; $^{13}$C NMR (100 MHz, $CD_3OD$) $\delta$ 28.1, 30.1, 33.1, 74.9, 123.3, 124.6, 124.7, 125.8, 125.7, 126.5, 127.1, 127.4, 128.5, 129.8, 130.9, 131.4, 136.5, 152.7, 166.2 ppm; IR (neat, NaCl): 3523, 2925, 2854, 1739, 1674, 1629, 1457, 1379, 1245 cm$^{-1}$; ESI-HRMS: $m/z$ calculated for $[C_{50}H_{47}N_8O_2]^+$, 791.3816 found 791.3816.

### Study of the photophysical properties

A stock solution of **BPTG** (10 mM) in a mixed solution of pure DMSO and 10 mM HEPES buffer pH 7.4 (1 : 6 v/v) was prepared. The UV-vis absorption spectra were recorded in 1 cm quartz cells at wavelengths ranging from 225 nm to 600 nm at room

temperature. The fluorescence spectra were recorded from 365 nm to 665 nm at room temperature using an excitation wavelength of 342 nm. The fluorescence quantum yield of **BPTG** was calculated from the absorbance (below 0.05) by comparing it to standard quinine sulfate ($\Phi_F$ = 0.54) in 0.1 M $H_2SO_4$.[30] The standard curve was obtained by recording the florescence from dilutions of the stock solution of LPS ranging from 5 nM to 50 μM. Then, the optimized conditions for the LPS sensor were investigated.

### Fluorescence emission measurements

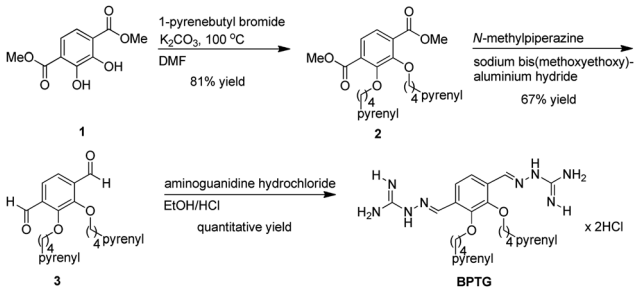
A stock solution of **BPTG** (100 mM) in pure DMSO was prepared. A stock solution of 1 mM LPS was prepared by dissolving in 10 mM HEPES buffer pH 7.4. A mixed solution of **BPTG** (100 mM, 0.5 mL) and LPS solution (1 mM, 50 mL) at room temperature was adjusted to 5.0 mL by adding the mixture of DMSO/10 mM HEPES buffer pH 7.4 (1 : 6 v/v) to afford final concentrations of 100 μM **BPTG** and 100 μM LPS. At a time-point 5 minutes after mixing, the fluorescence emission spectrum was recorded from 365 nm to 665 nm using an excitation wavelength of 342 nm. An interference study using other biological anionic species with this method utilized a stock solution of 10 mM of other common acid and phosphate species (malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate, lecithin, and heparin) in 10 mM HEPES buffer pH 7.4. Mixtures of **BPTG** (50 μM), LPS (5 μM) and each of these interfering species (50 μM) were prepared. The fluorescence emission spectra were recorded at the single wavelength of 485 nm, using an excitation wavelength of 342 nm. All measurements were taken after 5 minutes of mixing at room temperature.

### Computational experiments

Different conformations of the bispyrenyl bis-guanylhydrazone were obtained from the conformational search in Schrödinger software by using the ConfGen method with the OPLS_2005 force field. The maximum number of examined conformations was set to 1000. The DFT calculations with M06-2X functional provided the stable local minima of the **BPTG** clusters, namely sandwich-, semi- and open-stacking based on the folding of pyrene to bis-guanylhydrazone. All structures were optimized with a Gaussian 09 program. The 6-31G(d,p) basis set was used for all atoms. The molecular orbitals and charges were calculated with natural bond orbital (NBO) analysis.

## Results and discussion

A novel fluorescent probe to detect LPS based on bispyrenyl terephtalaldehyde-bis-guanylhydrazone (**BPTG**) was prepared by a straightforward three-step sequence in high yield. 2,3-Dihydroxyterephthalic acid dimethyl ester was alkylated with 1-pyrenebutyl bromide using $K_2CO_3$ as the base in DMF to give **2** in 81% yield (Scheme 1). Aldehyde **3** was obtained in 67% yield by the partial reduction of **2** with sodium bis(methoxyethoxy)-aluminium hydride (Red-Al) in the presence of *N*-methylpiperazine.[31] **BPTG** was obtained from the condensation of terephtaldehyde **3** and the hydrochloride salt of aminoguanidine in refluxing ethanol and hydrochloric acid. Pure **BPTG** was isolated as the crystalline hydrochloride salt after cooling to 0 °C and washing with cold ether.




Scheme 1 Synthesis of the LPS sensor **BPTG**.

Surprisingly, upon the addition of LPS into the **BPTG** solution, green excimer fluorescence (peaked at 485 nm) was observed with a characteristic feature of excimer emission along with an increase in monomer emission at around 375 nm (Fig. 2). The enhancement of both excimer and monomer emissions of the bispyrenyl system is not usual since most sensors based on the two pyrene unitslinked covalently to a central molecule show a ratiometric fluorescence response with the changes in excimer–monomer emissions, which involves the reduction of the monomer band and the increase in the excimer emission upon binding to targets and *vice versa*. Although the bispyrenyl molecule tends to form an excimer in an aqueous medium due to the lipid nature of pyrene,[18,19,32–35] it is known that aromatic compounds can intercalate to quench the excimer–monomer emissions.[36–38] Therefore, the molecular orientation of **BPTG** could play an important role in the fluorescent quenching process prior to the addition of LPS.

In order to better understand the structural behavior of **BPTG**, a conformational search was done to obtain the low-lying stable structures at the ground state of **BPTG** using the ConfGen[39] method with the OPLS_2005 force field in Schrödinger software. Three distinct conformations of **BPTG** as shown in Fig. 3, namely

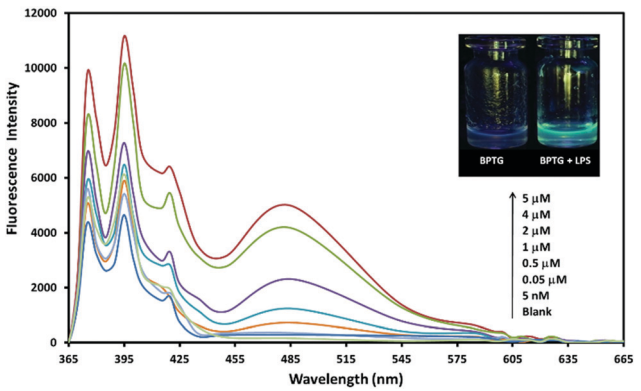



Fig. 2 Changes in the fluorescence emission spectra of **BPTG** (10 μM) upon the addition of LPS in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4). Excitation wavelength: 342 nm. (inset) Photograph of **BPTG** (10 μM) in the presence of 5 μM LPS in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4).

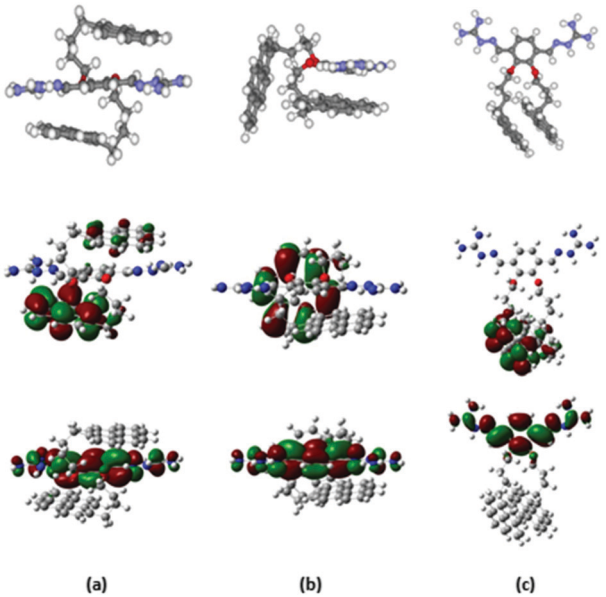
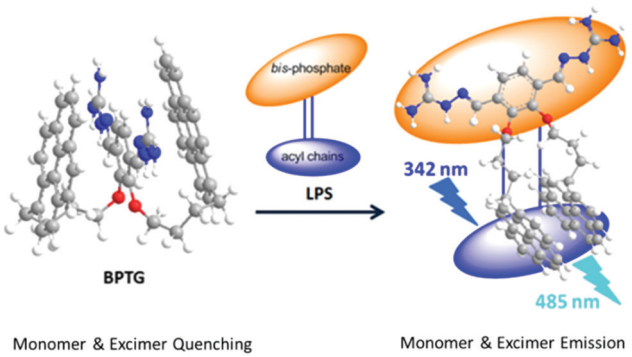



Fig. 3 Conformations of **BPTG**, HOMO (second row) and LUMO (third row) of (a) sandwich- (b) semi- and (c) open-stacking conformations optimized with the M06-2X functional in Gaussian 09 program.




Scheme 2 Proposed mechanism of the "turn-on mode" of **BPTG** after the addition of LPS.

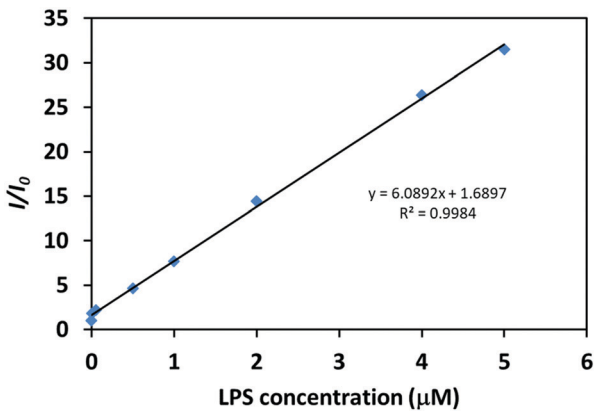



Fig. 4 Relative fluorescent signal ($I/I_0$) of **BPTG** ($I_{485}$ upon addition of LPS 0–5 μM, $\lambda_{ex}$ = 342 nm) in a solution of DMSO/HEPES buffer (10 mM, pH 7.4).

sandwich-, semi- and open-stacking based on the folding of pyrene to bis-guanylhydrazone, were later optimized using the DFT calculations with M06-2X functional[40–42] in the Gaussian 09 program to provide the stable local minima. The relative energies are 0.0, 4.4 and 15.5 kcal $mol^{-1}$ for sandwich-, semi- and open-stacking conformations, respectively.

A parallel and co-facial alignment of sandwich stacking conformation with a highly congruent HOMO and LUMO in Fig. 3 suggests that a pairing formed between the pyrenes and the bis-guanylhydrazone prevents the formation of a pyrene excimer in aqueous buffer *via* special π stacking with a butterfly-like skeleton. The distances between each pyrenyl unit and the phenyl ring of the terephtalaldehyde-bis-guanylhydrazone are in the range of 3–4 Å. The HOMO density of the π-electron donor is mainly located on one pyrenyl ring; moreover, the LUMO density of the π-electron acceptor is mainly located on the bis-guanylhydrazone system in the center part of the sandwich stacking. These molecular characters could ultimately play an important role in the fluorescence quenching of monomer and excimer emissions through photoinduced electron transfer (PET)[43] prior to the binding with LPS.

On the basis of theoretical studies, the plausible mechanism of **BPTG** in the present system is schematically depicted in Scheme 2 and accounts for the excimer–monomer emission 'turn-on' in aqueous solution. Upon the binding of LPS and **BPTG**, the aminoguanidine groups form salt bridges with the phosphate groups of LPS; moreover, the pyrene units of **BPTG** interact with the acyl chains of LPS *via* the lipophilic interactions. Therefore, it is most likely that the pyrenyl units are loosened off from the sandwich stacking and drawn into close proximity to form a pair of excimers with monomer and excimer emissions.

Upon the addition of LPS (5 μM), **BPTG** (10 μM) exhibited the highest $I/I_0$ ratio of about 30 times as shown in Fig. 4 and the quantum efficiency increased to 25% due to a conformational change, allowing excimer formation (Table S1, ESI†). The detection limit of **BPTG** for LPS was measured using the relative fluorescent signal plot ($I/I_0$) against LPS concentrations ranging from 0 to 5 μM, and this plot provided a good linear correlation (Fig. 4). The limit of detection determined by the plot was 5 nM under our experimental conditions. In order to understand the effect of the stoichiometric ratio of **BPTG** to LPS, we performed a Job's plot analysis, and the result suggested that the stoichiometric ratio of **BPTG** to LPS was 4 : 1 (Fig. 5).

To better investigate the practical applicability of **BPTG** as a LPS fluorescence sensor, competitive experiments were performed in the presence of LPS mixed with malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate, lecithin, and heparin, as shown in Fig. 6. No significant interference was observed in the detection of LPS with the **BPTG** sensor in the presence of the most competitive acid and phosphate biological species. Accordingly, these observations suggest that the **BPTG** sensor can be used as a selective fluorescent sensor for LPS in the presence of most biological anionic species.

Heparin is regarded as one of the clinically important medicines for preventing blood clots during heart surgery or for the treatment of certain bleeding disorders. **BPTG** showed a

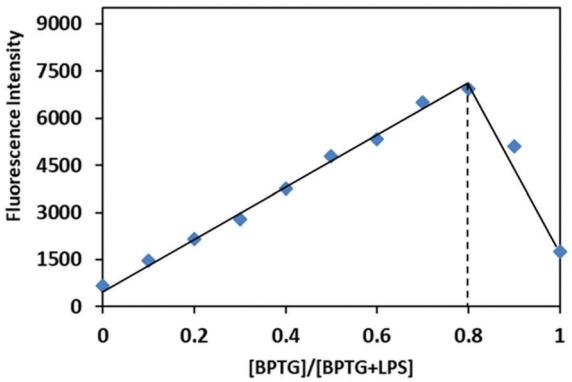



Fig. 5 Job's plot for the relationship between the fluorescence intensity of **BPTG** (100 μM) in DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4) and the mole fraction of LPS ($\lambda_{em}$ = 485 nm).

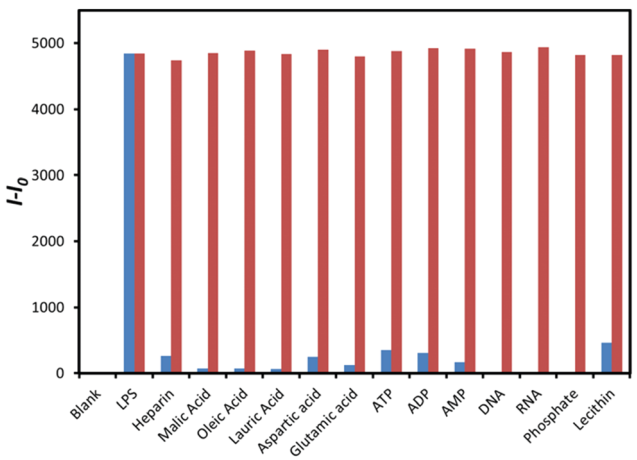



Fig. 6 Selectivity of **BPTG** (10 μM) to some biologically important anionic species (50 μM) in a DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4): heparin, malic acid, oleic acid, lauric acid, aspartic acid, glutamic acid, ATP, ADP, AMP, DNA, RNA, phosphate and lecithin. Blank refers to free **BPTG** (10 μM) in a DMSO/HEPES (v/v = 1/6) buffer solution (10 mM, pH 7.4).

slight change in fluorescence at 485 nm at high concentration (50 μM) due to the lack of alkyl chains. To the best of our knowledge, **BPTG** is the first chemosensor among other reported small molecule probes that can sense LPS over heparin. A small enhancement in the fluorescence emission at 485 nm was observed with lecithin since it possesses long alkyl chains and the negatively charged phosphate that can interact with the aminoguanidine moieties of **BPTG**.

To demonstrate the application of **BPTG** as a tool for biological studies, *Vibrio cholerae*, which are Gram-negative and virulent bacteria causing diarrhoeal disease, were incubated with **BPTG**. Green fluorescence was observed with a clear black background to reveal living bacteria colonies due to the expression of LPS on the cell surface, as shown in Fig. 7.

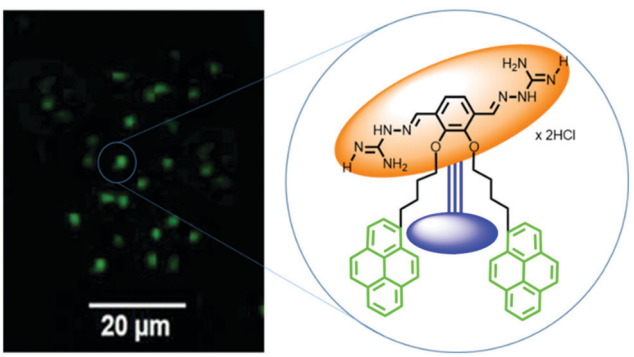



Fig. 7 Visualization of *V. cholerae* (~$10^6$ cells per mL) after incubation with **BPTG** (10 μM). Cells were imaged under Olympus FV10i at 100× magnification.

## Conclusions

In summary, the development of **BPTG** for LPS sensing led us to discover the unique molecular feature of **BPTG** as a self-quenching molecule prior to binding to LPS. **BPTG** is a turn-on probe for the LPS detection and displays monomer and excimer emissions in aqueous solution with the lowest detection limit at 5 nM. **BPTG** is highly selective for LPS over heparin and other anionic biological species. The unique molecular architecture of **BPTG** provides a better understanding for the rational design of fluorescence sensors.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

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Electronic Supplementary Material (ESI) for New Journal of Chemistry.


SUPPORTING INFORMATION

# Turn-on fluorescent sensor for the detection of lipopolysaccharide based on a novel bispyrenyl terephtalaldehyde-bis-guanylhydrazone

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## 1. Spectroscopic data of synthesized compounds

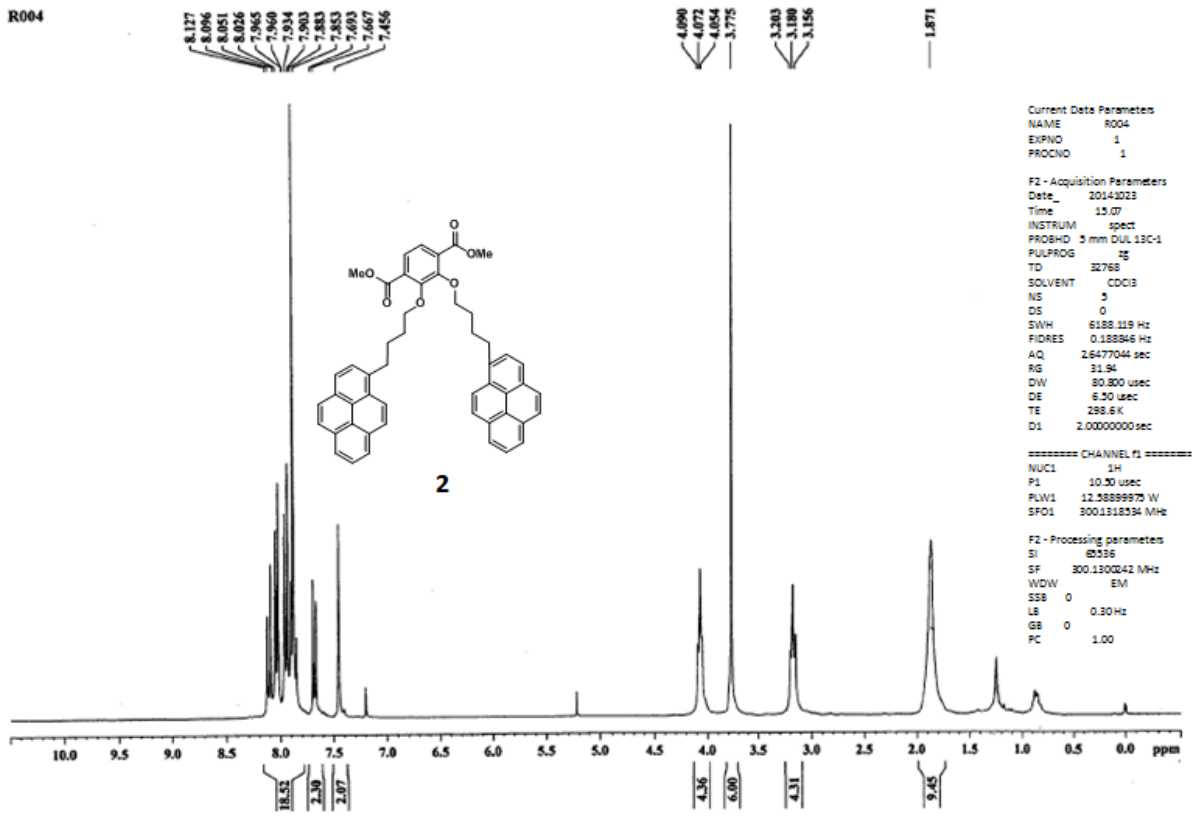



**Fig. S1** $^{1}$H NMR spectrum of dimethyl 2,3-bis(4-(pyren-1-yl)butoxy)terephthalate (**2**)

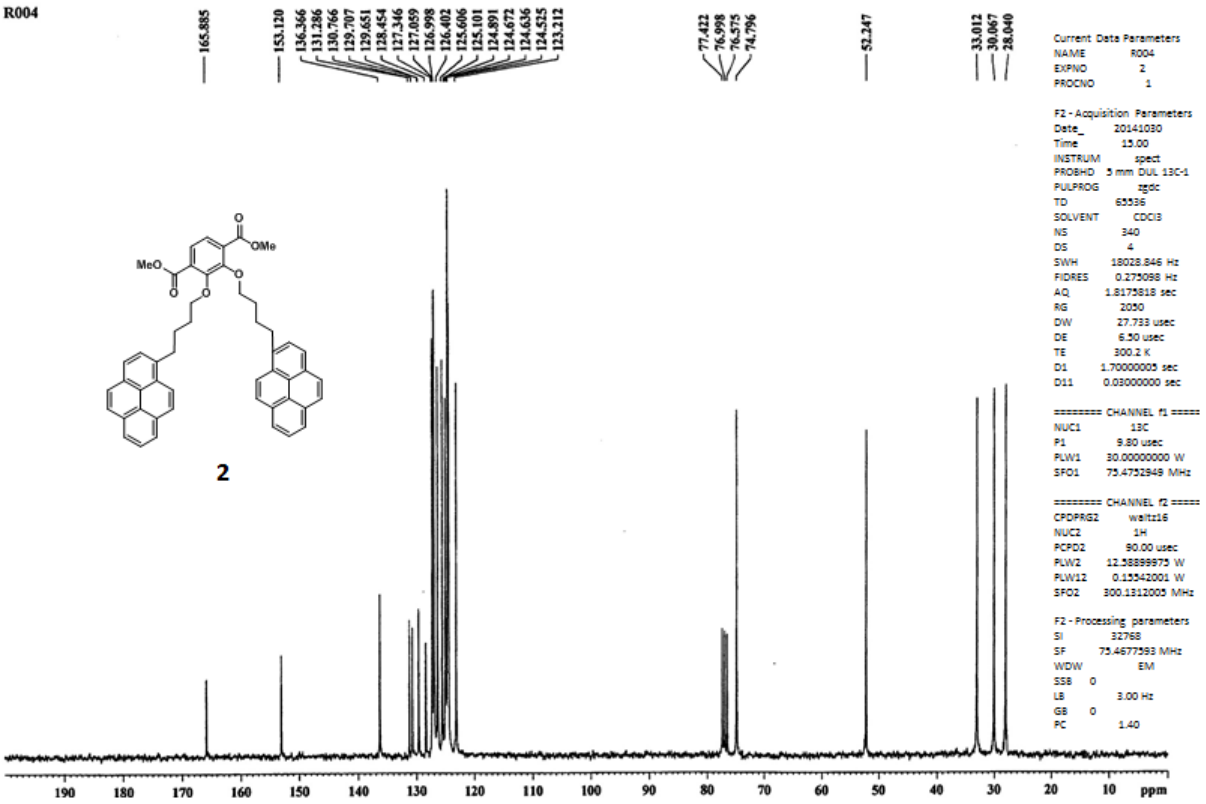



**Fig. S2** $^{13}$C NMR spectrum of dimethyl 2,3-bis(4-(pyren-1-yl)butoxy)terephthalate (**2**)

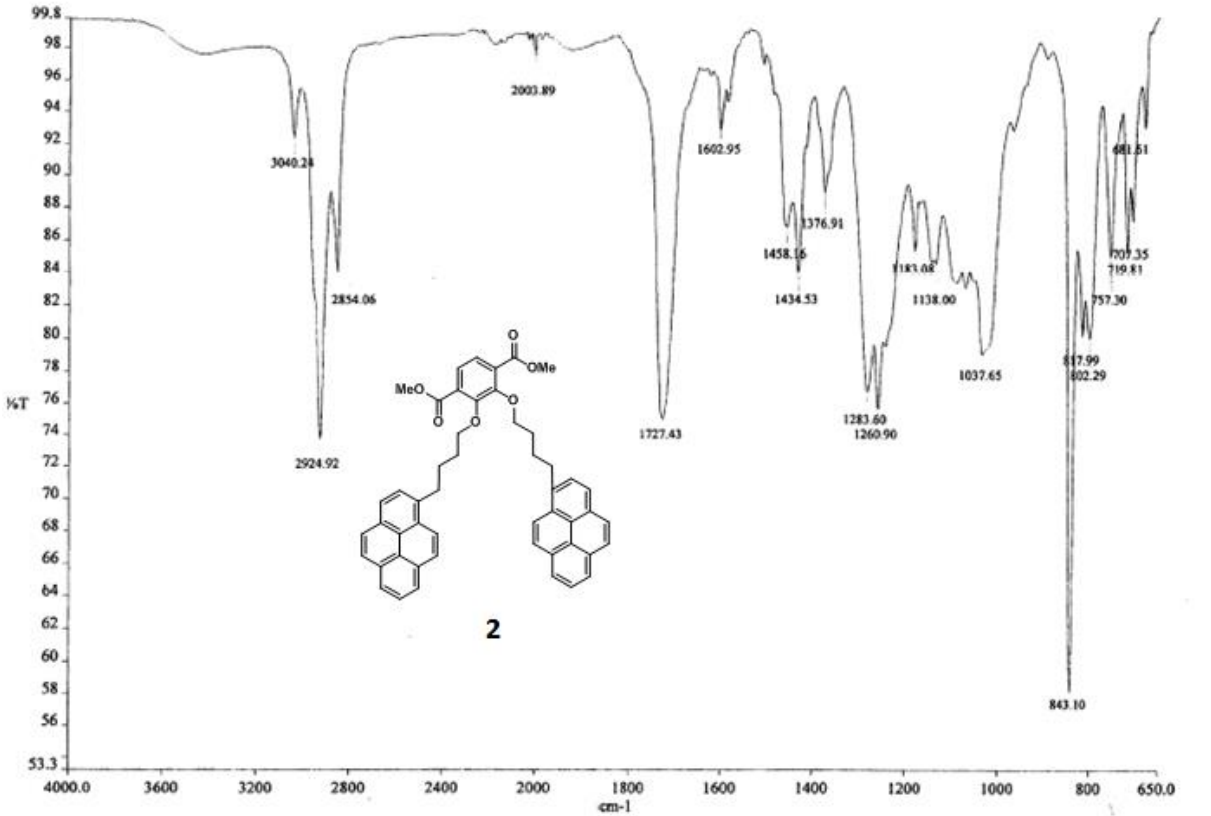



**Fig. S3** IR spectra of dimethyl 2,3-bis(4-(pyren-1-yl)butoxy)terephthalate (**2**)

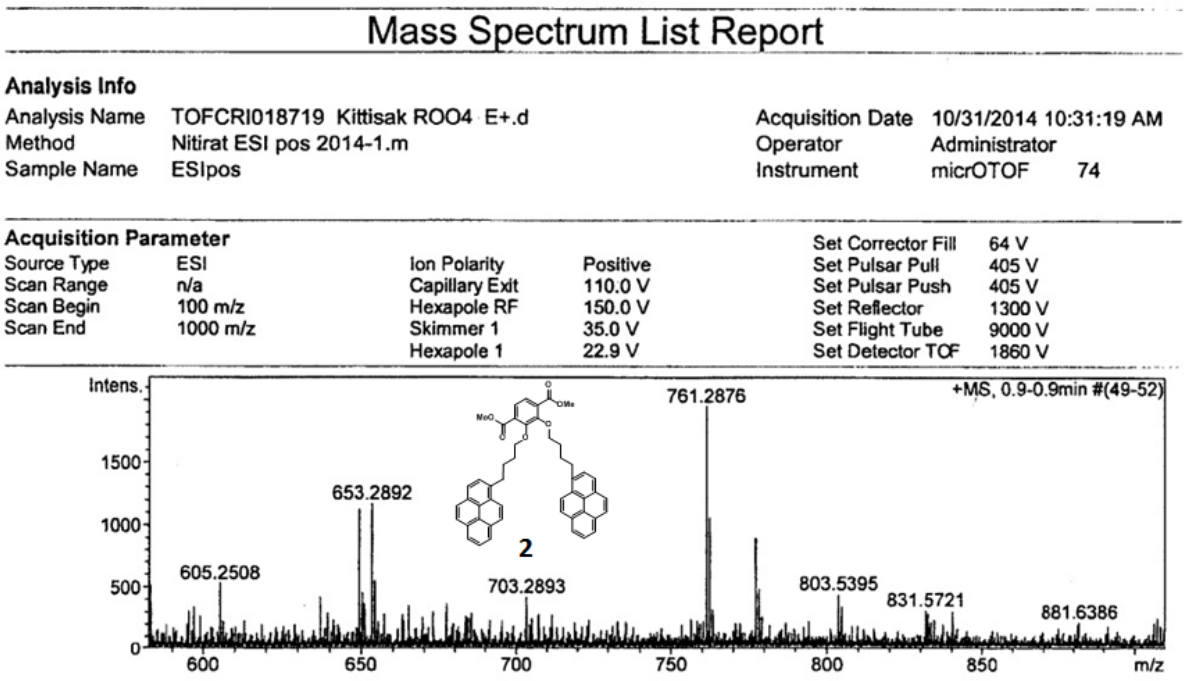



**Fig. S4** ESI-MS of dimethyl 2,3-bis(4-(pyren-1-yl)butoxy)terephthalate (**2**)

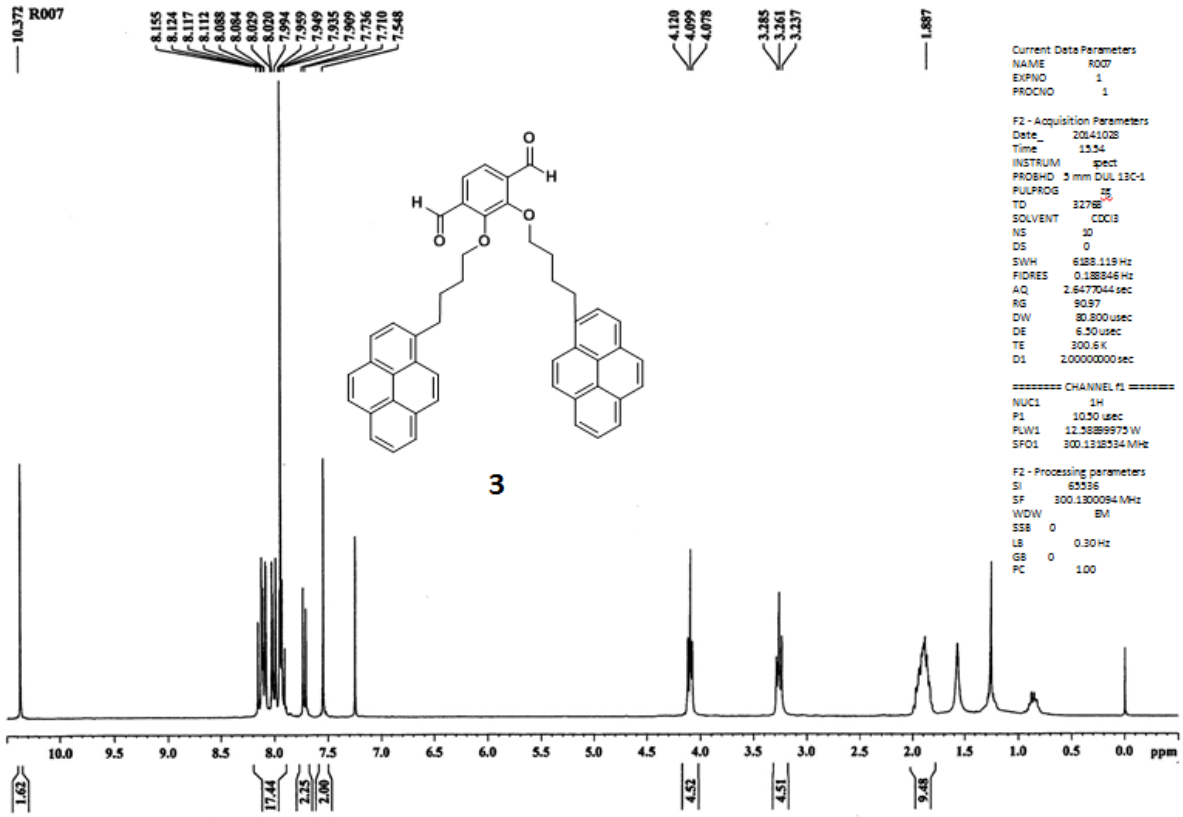



**Fig. S5** $^{1}$H NMR spectrum of 2,3-bis(4-(pyren-1-yl)butoxy)terephthalaldehyde (**3**)

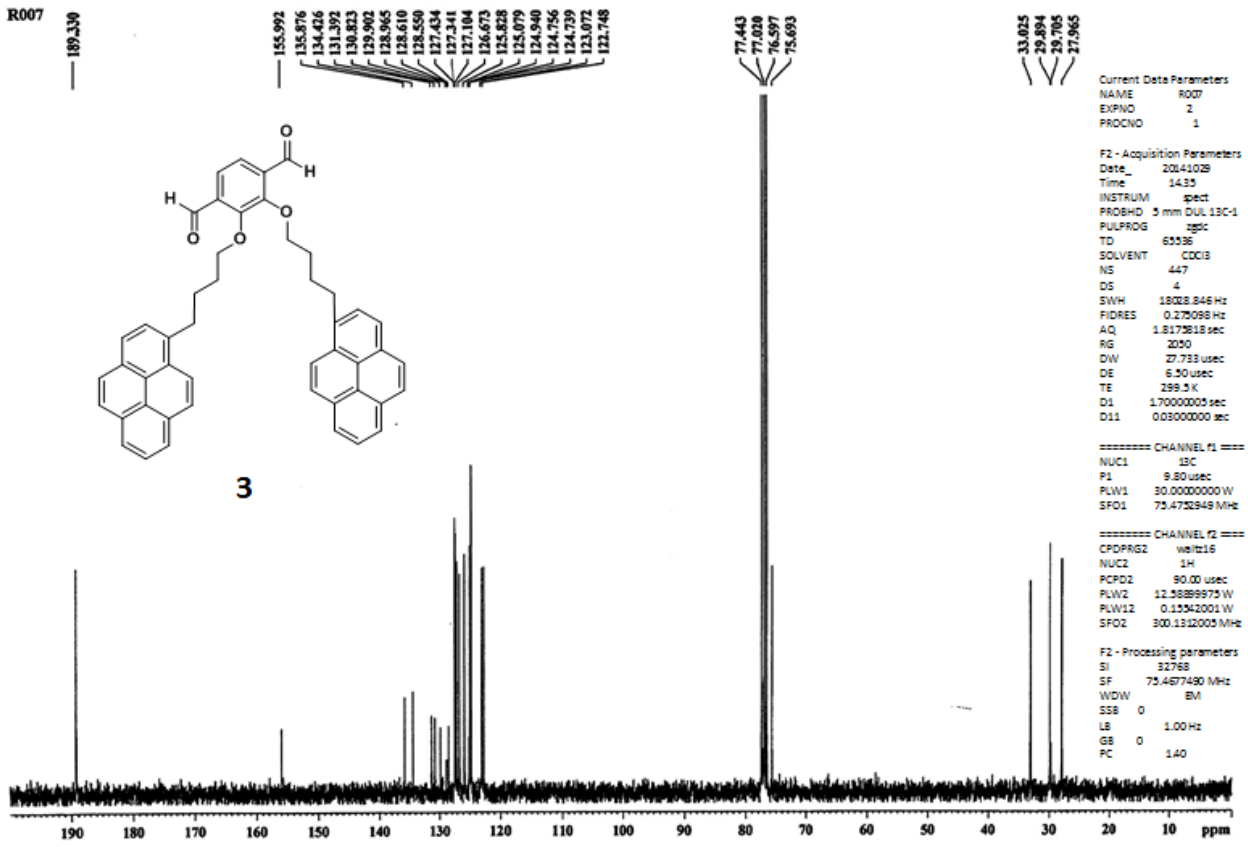



**Fig. S6** $^{13}$C NMR spectrum of 2,3-bis(4-(pyren-1-yl)butoxy)terephthalaldehyde (**3**)

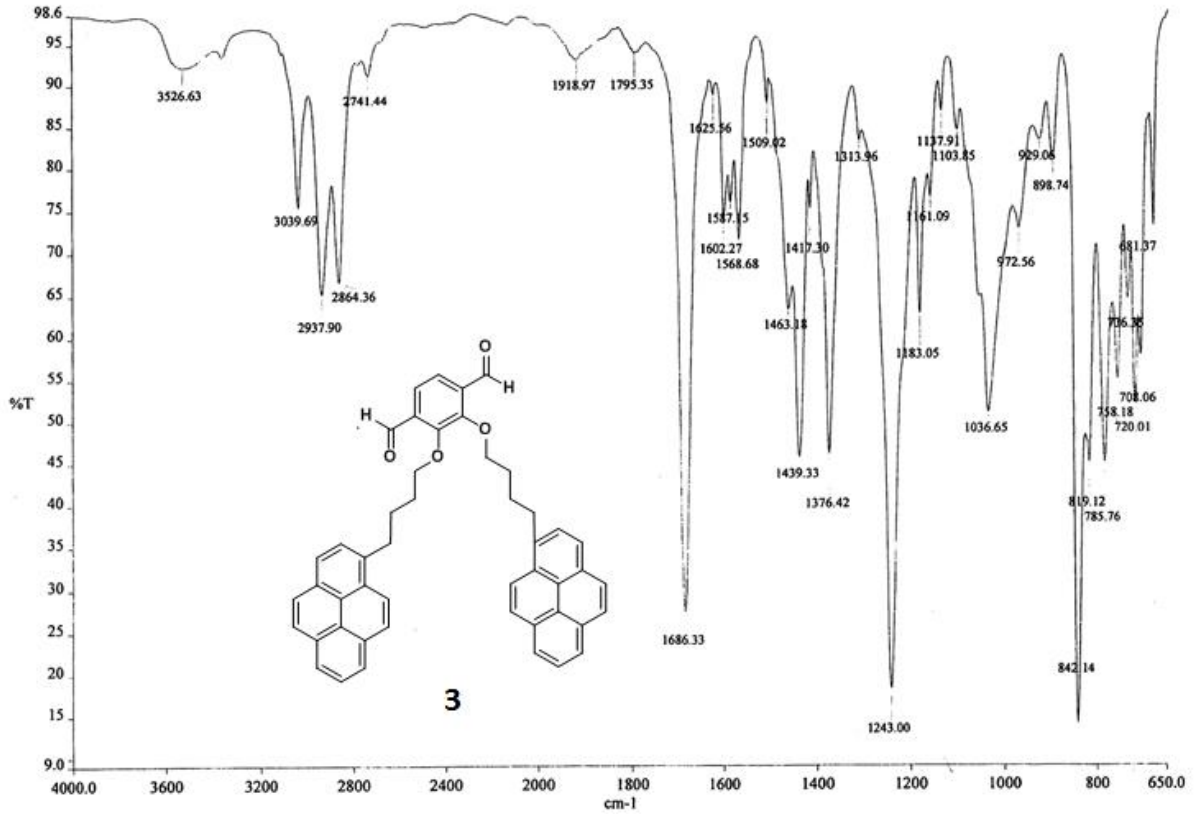



**Fig. S7** IR spectra of 2,3-bis(4-(pyren-1-yl)butoxy)terephthalaldehyde (**3**)

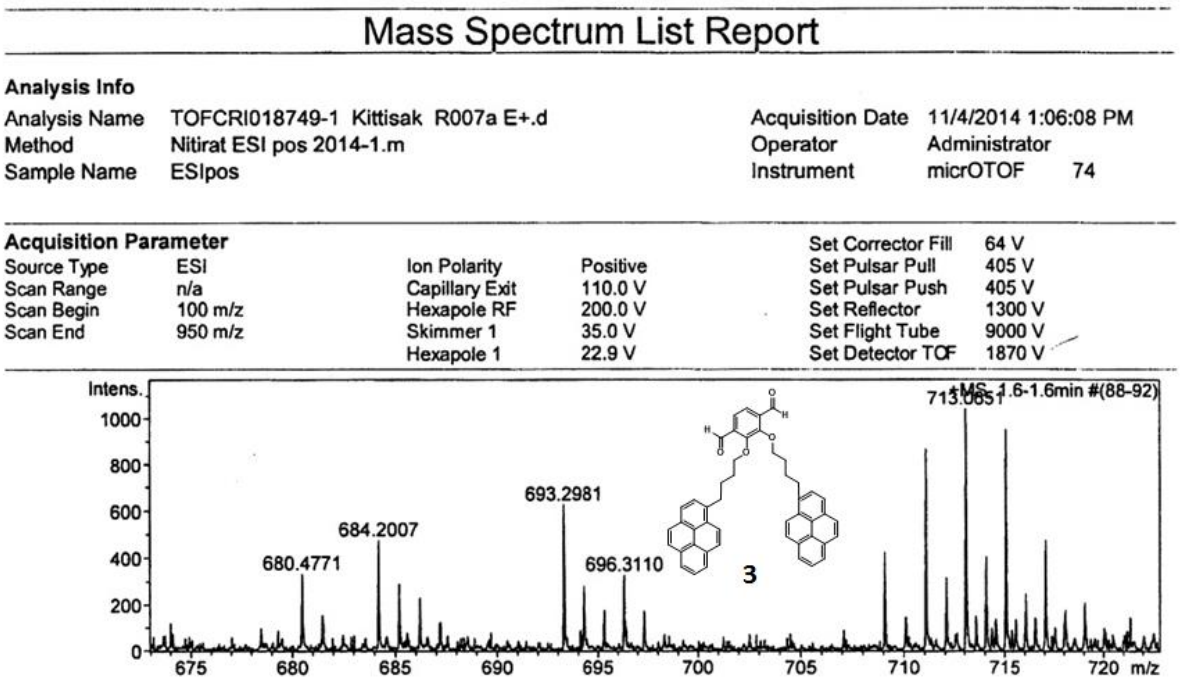



**Fig. S8** ESI-MS of 2,3-bis(4-(pyren-1-yl)butoxy)terephthalaldehyde (**3**)

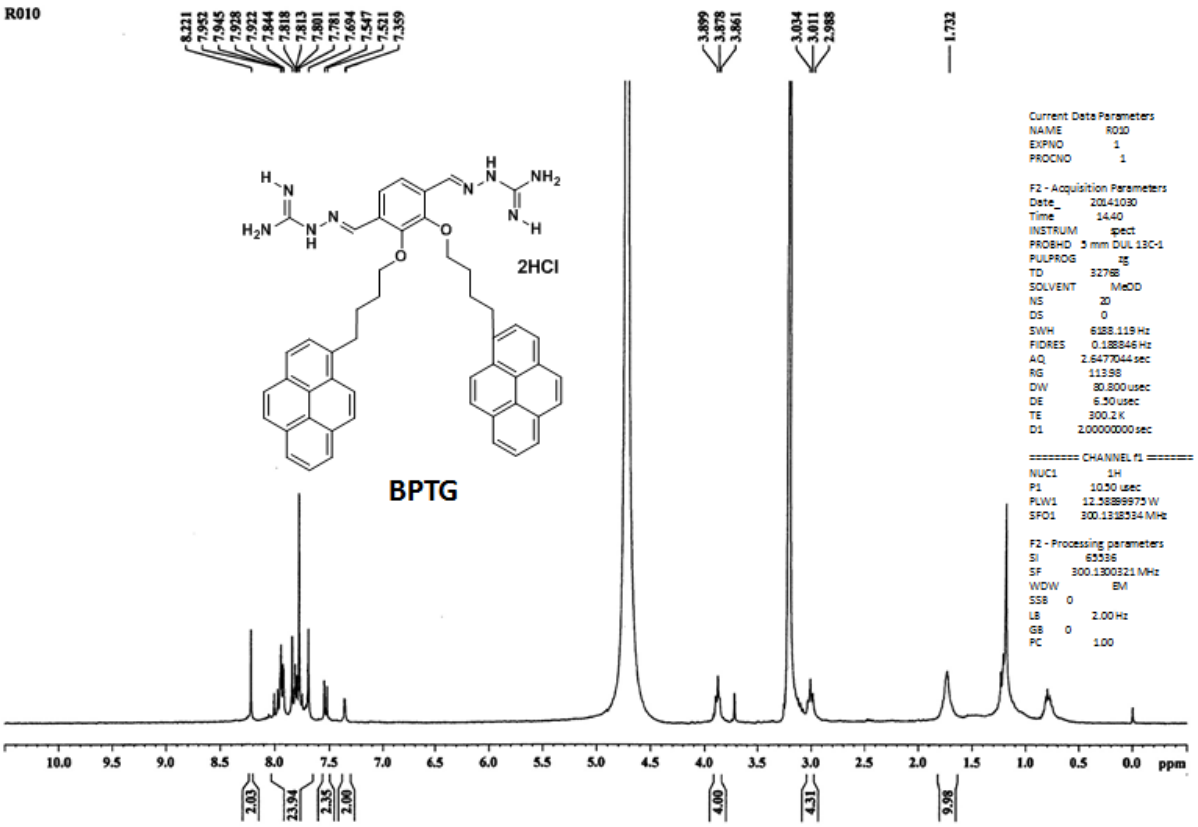



**Fig. S9** $^{1}$H NMR spectrum of 2E,2'E)-2,2'-((2,3-bis(4-(pyren-1-yl)butoxy)-1,4-phenylene)bis(methanylylidene))bis(hydrazinecarboximidamide) (**BPTG**)

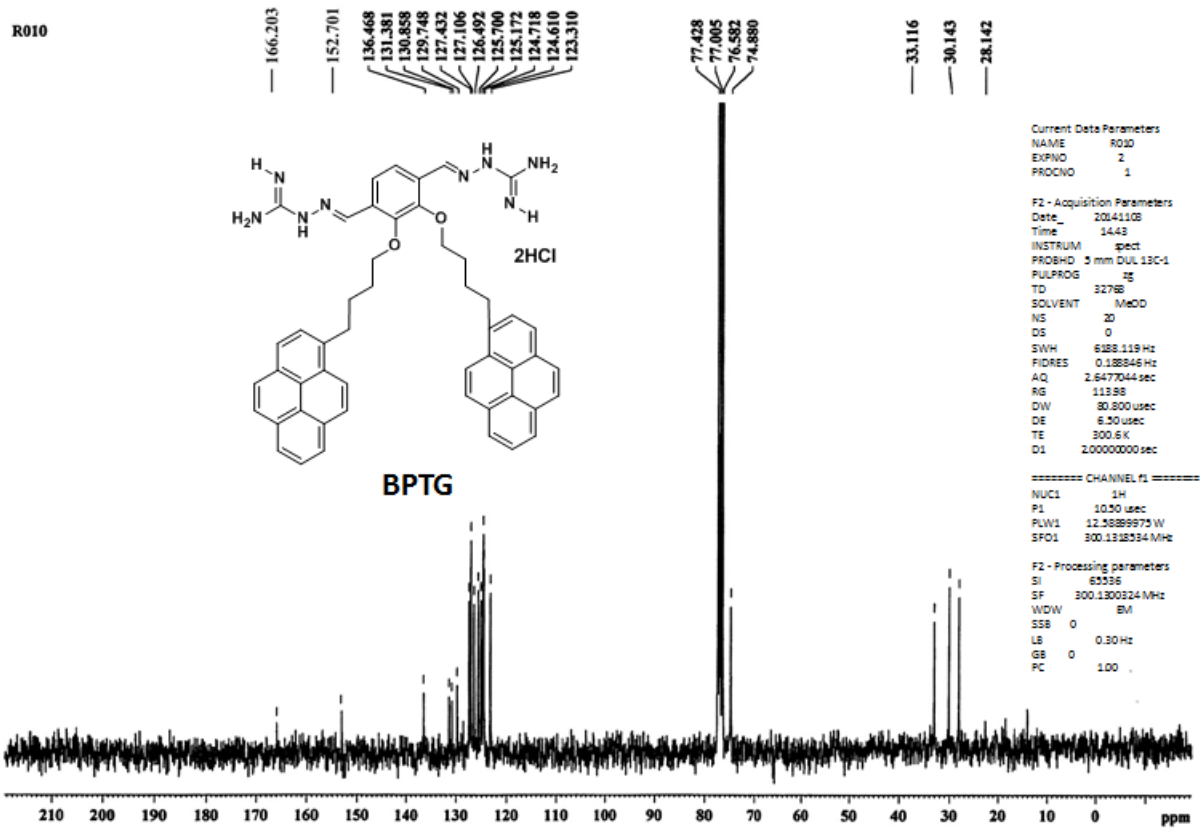



**Fig. S10** $^{13}$C NMR spectrum of 2E,2'E)-2,2'-((2,3-bis(4-(pyren-1-yl)butoxy)-1,4-phenylene)bis(methanylylidene))bis(hydrazinecarboximidamide) (**BPTG**)

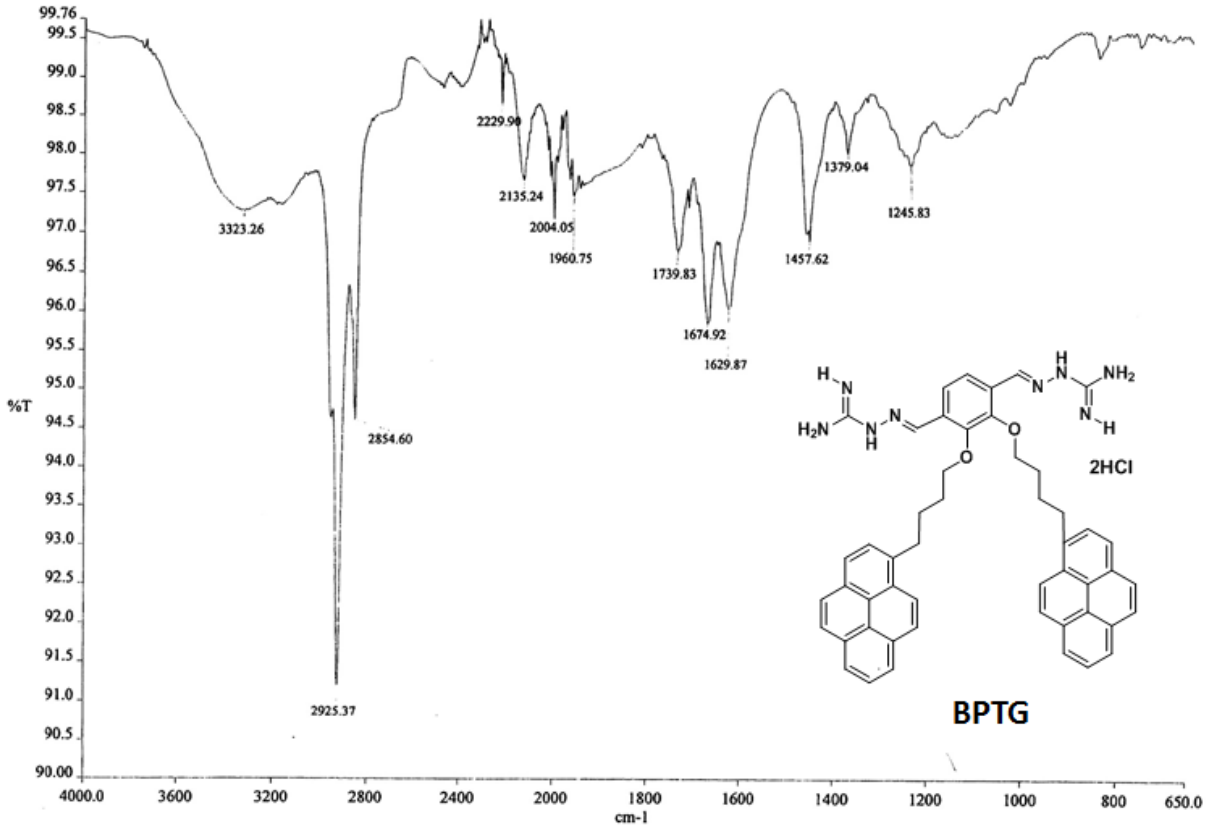



**Fig. S11** IR spectra of 2E,2'E)-2,2'-((2,3-bis(4-(pyren-1-yl)butoxy)-1,4-phenylene)bis(methanylylidene))bis(hydrazinecarboximidamide) (**BPTG**)

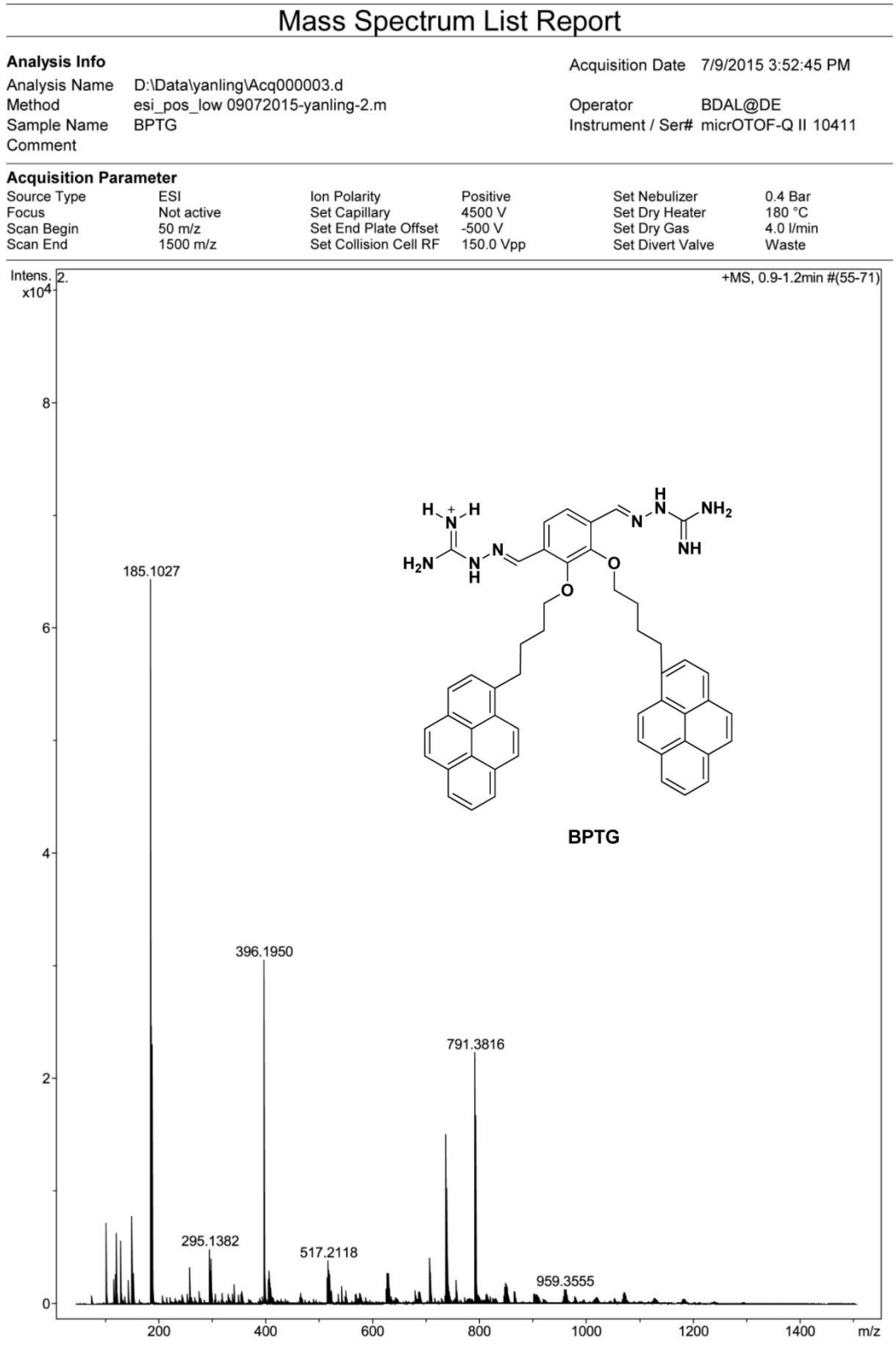



**Fig. S12** ESI-MS of 22E,2'E)-2,2'-((2,3-bis(4-(pyren-1-yl)butoxy)-1,4-phenylene)bis(methanylylidene))bis(hydrazinecarboximidamide) (**BPTG**)

**2. Absorption spectra of BPTG before and after the addition of LPS**

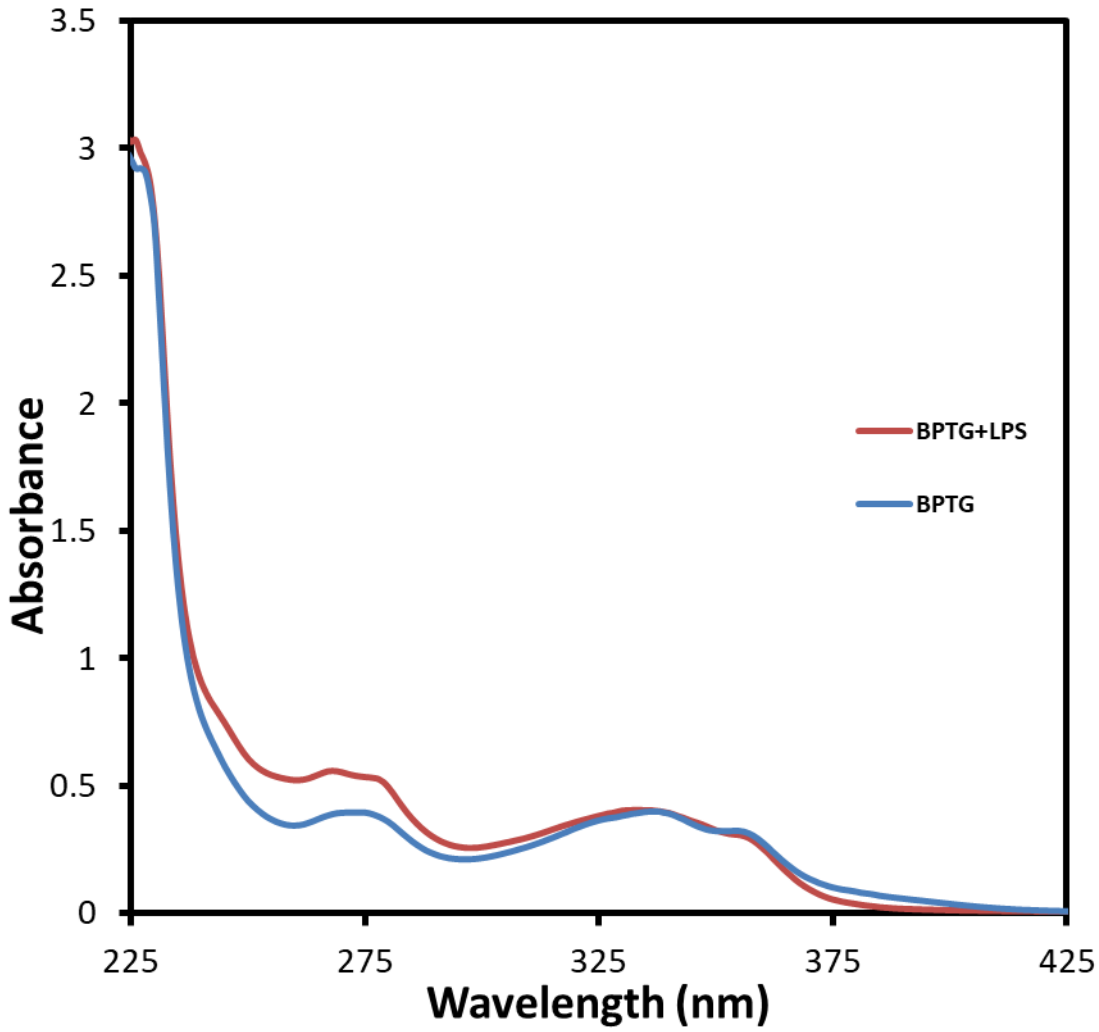



**Fig. S13** Absorption spectra of **BPTG** (5 μM) in a solution of DMSO/HEPES (v/v = 1/6) buffer (10 mM, pH 7.4) before and after addition of LPS (10 μM). The UV-vis absorbance maximum at 342 nm is corresponding to the pyrenyl band.

**3. The photophysical properties of BPTG**

**Table S1.** Photophysical properties of BPTG in a solution of DMSO/HEPES (v/v = 1/6) buffer (10 mM, pH 7.4) before and after addition of LPS.

| Compound | Absorption | | Emission | |
|---|---|---|---|---|
| | $\lambda_{max}$(nm) | ε ($M^{-1}cm^{-1}$) | $\lambda_{em}$(nm) | $\Phi_F$[a] |
| **BPTG** | **342** | **76,320** | **485** | **0.072** |
| **BPTG + LPS** | **342** | **78,537** | **485** | **0.090** |

**a** : Quinine sulfate in 0.1 M $H_2SO_4$ ($\Phi_F$ = 0.54) was used as standard

## 4. Results of computational experiments

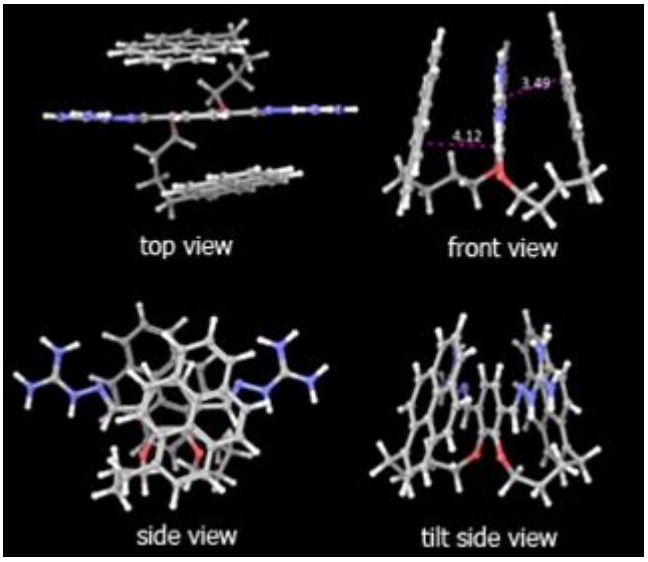
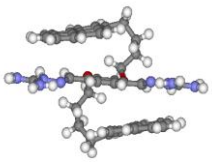
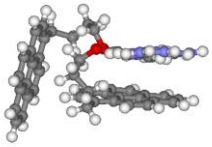
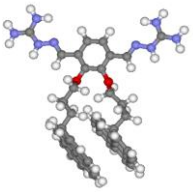



**Fig. S14** Sandwich stacking conformation of **BPTG** like a butterfly-like skeleton

**Table S2. BPTG**'s conformations and their total energies, HOMO's enegies, LUMO's enegies and band gap of (a) sandwich- (b) semi- and (c) open-stacking optimized with the M06-2X functional in Gaussian 09 program.

| **BPTG's Conformation** |  |  |  |
|---|---|---|---|
| | **sandwish-** | **semi-** | **open-** |
| Total Energy (au) | -2521.337149 | -2521.330214 | -2521.312463 |
| Relative Energy (kcal/mol) | **0.00** | **4.35** | **15.49** |
| LUMO (au) | -0.21554 | -0.22098 | -0.23608 |
| HOMO (au) | -0.38725 | -0.36041 | -0.32508 |
| band gap (eV) | 4.67257252 | 3.79416916 | 2.421868 |

The M06 functional was developed by Zhao and Truhlar. [1-3] The M06 functional, which take care the van der Waals interaction, has been successful studied on the adsorption and reaction on several material such as zeolite,[4] alloy metal,[5] porphyrin[6] and metal-organic framework.[7] We optimized all structures with Gaussian 09 program.[8]

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