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# Tetraphenylethylene-based Glycoconjugate as a Fluorescence "Turn-On" Sensor for Cholera Toxin

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**Abstract:** Tetraphenylethylene (TPE)-based glycoconjugates were easily synthesized by copper(I)-catalyzed "click reactions" between propargyl-attached TPE and azido-functionalized sugars. The TPE compound bearing lactosyl moieties (**Lac-TPE**) was found to be a fluorescence "turn-on" sensor for cholera toxin by virtue of aggregation-induced emission characteristics of the TPE motif owing to the specific interaction of lactose with the cholera toxin B subunit, whilst a cellobiose-functionalized TPE derivative did not show any response to the toxin. Therefore, **Lac-TPE** shows promising applications in the detection of cholera toxin, as well as in the investigation of carbohydrate–protein interaction.

**Keywords:** aggregation-induced emission · cholera toxin · lactose · tetraphenylethylene

## Introduction

Cholera continues to represent a major threat to both human and animals, especially in developing countries. Cholera is an acute intestinal infection, characterized by profuse watery diarrhea and vomiting, which can lead to severe dehydration, electrolyte imbalance, and even death if treatment is not given appropriately.[1] Cholera toxin (CT), the primary virulence factor of cholera, is secreted by the bacterium *Vibrio cholerae* and possesses an $AB_5$ hexameric architecture. The five identical B-subunits of such toxin bind selectively to the glycolipid ganglioside GM1 that contains a pentasaccharide. It is the recognition of this pentasaccharide that facilitates the initial attachment of cholera toxin B-subunit (CTB) to the intestinal cells, which is the first step towards contracting the disease cholera. CTB is known to bind to lactose through the recognition of the terminal galactose portion of the molecule,[2] which is also the terminal sugar in the pentasaccharide headgroup of GM1 ganglioside.[3]

It is well-known that carbohydrate-mediated multivalent interactions play important roles in numerous biological processes, such as cell-growth and -recognition, bacterial and viral infections, inflammation, and cancer metastasis.[4] The specificity and affinity of these interactions depend strongly on multivalency, owing to the well-known "cluster glycoside effect".[5] Because most sugar ligands weakly bind to their protein acceptors, multivalency in carbohydrate–protein interactions becomes a prevalent principle to improve the specificity and affinity.[6] Lactosyl-bearing multivalent systems, such as dendrimers[2b] and nanoparticles,[2c] have been shown to possess specific binding to CTB, in which the specific interactions were assayed by fluorescence-quenching and red-shift of the adsorption, respectively.

Recently, molecules with aggregation-induced emission (AIE) characteristics have provided a promising platform in applications ranging from optical materials to sensors, owing to their enhanced emission in their aggregate or solid-state forms.[7] The AIE effect can significantly improve the fluo-

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rescence quantum yields of the molecules by up to two orders of magnitude, and enhance the photoluminescence intensity from faint luminophores into strong emitters.[8] Of these AIE molecules, TPE can be readily produced from a facile synthesis and shows an enhanced emission in both its aggregated form and the solid state.[9] In contrast to aggregation-caused fluorescence-quenching of conventional dyes, TPE-based AIE-active materials improve efficiency and sensitivity as chemosensors[10] or bio-probes[11] and have already shown practical applications in these fields. For example, Zhang and co-workers demonstrated that the TPE compounds bearing adenine and thymine moieties can be used as chemosensors for silver(I) and mercury(II) ions, respectively.[10b] In addition, Tang and co-workers synthesized diphenylated TPE dyes and found that the emission of such dyes could be switched off and on reversibly by wetting and dewetting with solvent vapor (e.g. $CHCl_3$), thereby manifesting their ability to optically sense volatile organic compounds.[10a] They also developed various types of TPE motifs containing charged groups, such as ammonium or sulfonate groups, which were used as turn-on luminescent probes for biomacromolecules, such as proteins and DNA.[9,11a,b] Kato et al. prepared a TPE-based fluorescent oligosaccharide probe bearing a 6′-sialyllactose moiety and utilized the probe as a "turn-on" fluorescent sensor for the detection of the influenza virus.[11e] Sanji and co-workers synthesized a series of sugar-modified TPEs, through which carbohydrate–protein and protein–protein interactions were investigated.[11f,g] Considering the specificity and affinity of carbohydrate–protein interactions, the grafting of carbohydrates onto TPE can give rise to new properties and potential applications as biosensors and in investigating carbohydrate–protein interaction.

Herein, we report the synthesis of a new AIE-active TPE derivative bearing lactose moieties and its potential application as a fluorescence sensor for CT. Four lactose moieties were readily introduced onto the TPE-derivative by a copper(I)-catalyzed "click reaction" between propargyl-attached TPE and azido-functionalized lactose. The introduction of four lactose moieties not only improves the water-solubility and biocompatibility, but also provides multiple binding sites towards CTB and enhances the binding through multivalent interactions. **Lac-TPE** becomes significantly luminescent when CTB is added, whilst cellobiose-functionalized TPE derivative (**Cel-TPE**) does not show the same phenomenon. Furthermore, we believe that the use of carbohydrate-bearing TPE as a fluorescence "turn-on" sensor provides a unique platform to investigate carbohydrate–protein interactions.

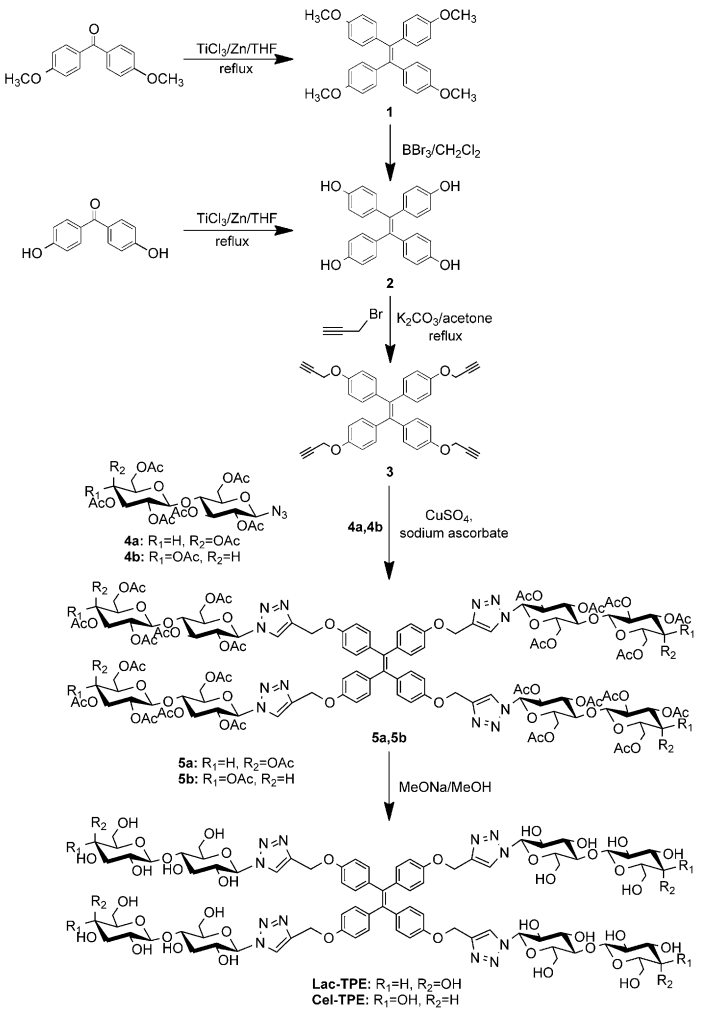
**Abstract in Chinese:**

通过炔丙基修饰的四苯乙烯和叠氮功能化的糖之间发生一价铜催化的"click"反应合成糖-四苯乙烯缀合物。利用乳糖与霍乱毒素 B 亚基的特异性相互作用和四苯乙烯的聚集诱导发光效应可以实现乳糖-四苯乙烯缀合物对霍乱毒素的荧光增强传感。而同样方法合成的纤维二糖-四苯乙烯缀合物对霍乱毒素无响应。因此，乳糖-四苯乙烯缀合物在霍乱毒素传感和糖–蛋白质相互作用研究中具有潜在应用价值。

## Results and Discussion

### Synthesis of Sugar–TPE Derivatives

Sugar–TPE derivatives were prepared as shown in Scheme 1. TPE derivative **2** was first prepared using the McMurry reaction[12] from 4,4′-dihydroxybenzophenone.




Scheme 1. Synthetic route to **Lac-TPE** and **Cel-TPE**.

However, the yield of this reaction was poor (only 34%), which is consistent with the result reported by Kato et al.[11e] Further investigation showed that McMurry coupling of 4,4′-dimethoxybenzophenone to produce compound **1** and then demethylation with $BBr_3$ can give **2** smoothly. Compared with the straightforward coupling of 4,4′-dihydroxybenzophenone, the yield of the two-step preparation was much higher (up to 84%). The poor yield of the one-step preparation may result from some side-reactions induced by the active hydroxyl groups during the McMurry reaction. Be-

sides characterization by NMR spectroscopy, compound **1** was further identified by single-crystal X-ray diffraction analysis. We found that the four phenyl rings of TPE were non-planar (Figure 1), owing to the steric effect of the peripheral phenyl rings. Efficient transformation from compound **1** into **2** was confirmed by the absence of resonance peaks of methoxy protons in the $^1$H NMR spectrum after the reaction had been completed. Further treatment of **2** with 3-bromo-1-propyne in the presence of $K_2CO_3$ afforded propargyl-attached TPE derivative **3**. Subsequently, lactosyl moieties were introduced onto TPE via copper(I)-catalyzed "click reactions"[13] between **3** and azido-functionalized lactose derivative **4a**, which was prepared according to previous procedures.[14] The formation of the triazole ring was confirmed by a chemical shift at 7.78 ppm (single peak, 4H) in $^1$H NMR spectrum and two peaks at 121.3 and 144.9 ppm in $^{13}$C NMR spectrum (see the Supporting Information). After the removal of all of the acetyl groups with $CH_3ONa$/$CH_3OH$, the desired water-soluble **Lac-TPE** was obtained in an excellent yield (97%). Compared with the protected precursor **5a**, the peaks of acetyl groups (2.14–1.95 ppm) in the $^1$H NMR spectrum disappeared and those for the hydroxy groups (5.16–4.57 ppm) of **Lac-TPE** appeared after efficient deprotection (see the Supporting Information). Analysis of the ESI-ion-trap mass spectrum of **Lac-TPE** revealed a peak at $m/z$ 2075.04 ($[M+H_2O+K]^+$). Similarly, **Cel-TPE** was also prepared and characterized for the control study.

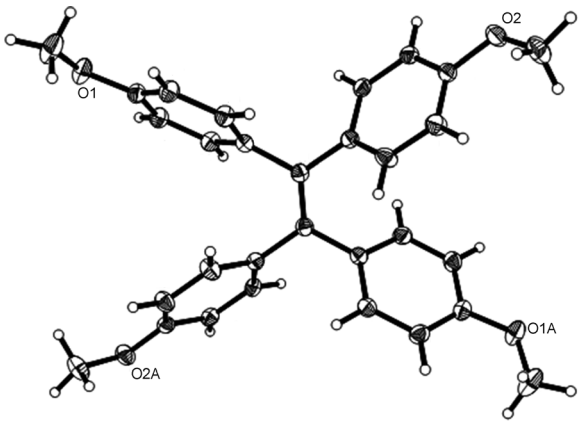



Figure 1. Crystal structure of TPE derivative **1**.[16]

### AIE effect of Lac-TPE

With **Lac-TPE** in hand, its AIE effect was studied firstly based on solvent-assistant aggregation. When dissolved in soluble solvents, such as water, **Lac-TPE** was nonluminescent in a relatively dilute solution. The fluorescence emission enhanced significantly with an increase in concentration of **Lac-TPE** in aqueous solution (Figure 2b). There may be two reasons for this enhanced fluorescence intensity, that is, the increased adsorption and the aggregation of **Lac-TPE** in water at a higher concentration. Figure 2a confirms the adsorption intensity increases with the concentration and the red-shift of up to 12 nm of the adsorption near 250 nm was attributed to the aggregation of the **Lac-TPE** molecules.

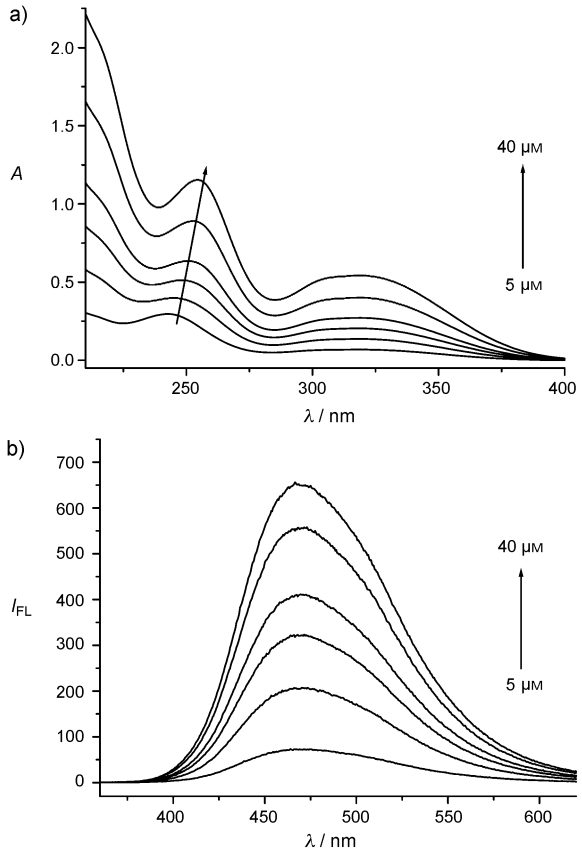



Figure 2. a) UV/Vis and b) fluorescence spectra for aqueous **Lac-TPE** solution of different concentrations (from 5 to 40 μM).

Addition of tetrahydrofuran or acetonitrile (even up to 99% in volume fraction) into the dilute aqueous solution of **Lac-TPE** did not dramatically improve its emission efficiency (see the Supporting Information, Figure S1). This phenomenon should be attributed to the amphiphilic properties of **Lac-TPE**, whose lactosyl groups are hydrophilic and TPE moiety is lipophilic. The Tang group has also obtained similar results with a TPE derivative containing amino groups that is hydrophilic.[11b] Therefore, a co-solvent system of dichloromethane (a poor solvent) and dimethyl sulfoxide (DMSO, a good solvent) was used to investigate the AIE effect of **Lac-TPE**. As shown in the Supporting Information, Figure S2, in the dilute dimethyl sulfoxide solution, **Lac-TPE** is nonemissive, regardless of addition of dichloromethane up to 80% (v/v). When the volume fraction of dichloromethane reached 85% or higher (the final concentration of **Lac-TPE** was kept constant at 3.7 μM), the fluorescence intensity increased significantly under the same measurement conditions owing to the aggregation of **Lac-TPE** in the dimethyl sulfoxide/dichloromethane solution, which leads to restricted intramolecular rotations and decreased nonradiative decay.[7c,d] The inset photos in the Supporting

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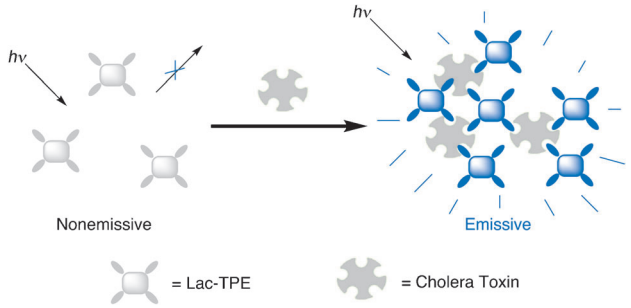
Information, Figure S2, show the enhanced photoluminescence intensity of **Lac-TPE** solution from a faint luminophore into a strong emitter when **Lac-TPE** aggregates. In addition, **Cel-TPE** showed a similar phenomena (see the Supporting Information, Figure S3).

### Fluorescence "Turn-On" Assay for Cholera Toxin

CT is a pentamer composed of five identical monomers, each with a binding site for the GM1 ganglioside.[3a] These binding sites, matching with the galactose moieties of the GM1 ganglioside, make it possible for CTB to bind to various types of molecules that have the same terminal galactose moiety. Lactose, as a disaccharide consisting of glucose and terminal-galactose units, was thus used to design CT sensors. The design rationale for the CT assay is illustrated in Scheme 2. **Lac-TPE**, possessing four lactosyl units, is expected to display weak photoluminescence in aqueous media. When CT was added to a dilute aqueous solution of **Lac-TPE**, multiple binding events occurred between the toxin and the multivalent lactosyl-attached TPE, leading to aggregation of **Lac-TPE**. As a result, the fluorescence of the ensemble would be increased significantly, which is induced by the aggregation of **Lac-TPE**.




Scheme 2. Illustration of fluorescence "turn on" assay for CT with **Lac-TPE** based on AIE effect.

Figure 3a shows the fluorescence spectrum of **Lac-TPE** and those in the presence of different amounts of CTB in PBS solution. **Lac-TPE** showed rather weak emission when its concentration was 2.0 μM. After the addition of CTB, the emission band around 475 nm emerged and its intensity increased gradually. The enhanced emission increased significantly when the concentration of CTB was below 2.0 μM, and the enhanced emission got less significant when the concentration of CTB was up to 5.0 μM, thereby indicating that the amount of CTB begins to be in excess. Photographs of solutions of **Lac-TPE** and the ensemble **Lac-TPE/CTB** in PBS buffer solution taken under illumination of a UV lamp are given in Figure 3a, left inset. The observed fluorescence enhancement is mainly caused by restricted intramolecular rotation of phenyl groups in the aggregated state, which blocks the nonradiative decay of **Lac-TPE** and makes it highly luminescent. The change of fluorescence intensity

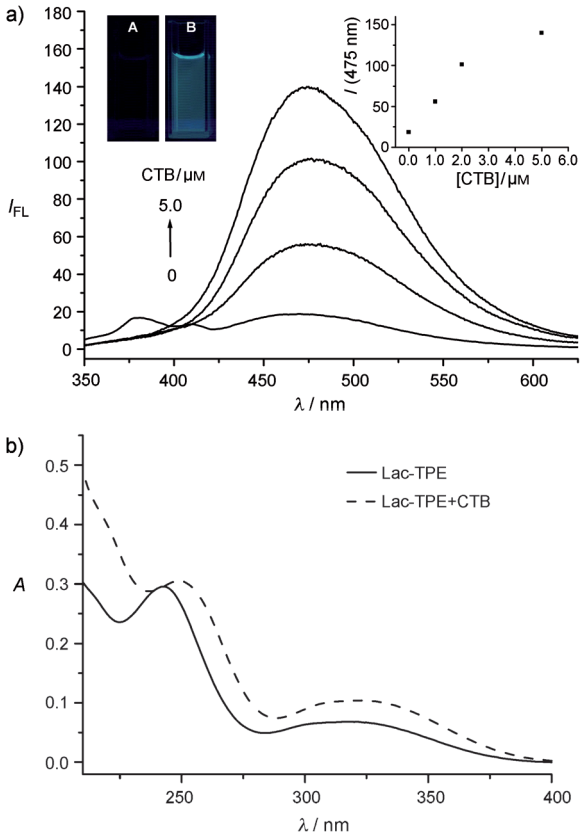



Figure 3. a) Fluorescence spectra of **Lac-TPE** (2.0 μM in 50 mM PBS solution, pH 7.3) in the presence of different amounts of **CTB** (0, 1.0, 2.0, and 5.0 μM); left inset: photos of the corresponding solutions of **Lac-TPE** in the absence (A) and presence of **CTB** (B; 5.0 μM) under UV light (365 nm) illumination; right inset: plot of the fluorescence intensity ($I_{475}$ nm) versus the concentration of CTB. b) UV/Vis spectra of **Lac-TPE** PBS solution in the absence and presence of **CTB** (5.0 μM).

Figure 3b). This result is consistent with both the aforementioned aggregation induced by the added concentration of **Lac-TPE** and the reported aggregation of dimethoxytetraphenyletheylene induced by the increasing poor solvent, which can verify the formation of the **Lac-TPE**/CTB ensemble.[11a]

became larger with increasing amounts of the toxin owing to the formation of more and larger aggregates. When the concentration of CTB was as low as 1.0 μM, the fluorescence emission of **Lac-TPE** was also obviously enhanced, up to three folds of that of **Lac-TPE** solution in the absence of CTB. Compared with previous reported CT sensors,[15] **Lac-TPE** has the advantages of a simple synthesis, without the need for complex instrumentation, though does show a lower sensitivity. Furthermore, the sensitivity is expected to increase when more lactosyl moieties are introduced onto TPE owing to the multivalent interactions.[2b,c]

In order to identify the formation of aggregates, the absorption spectra of **Lac-TPE** in the absence and presence of CTB were investigated. Compared to a **Lac-TPE** solution without CTB, the adsorption showed a red-shift of 8 nm (from 242 nm to 250 nm) when CTB (5.0 μM) was added to the **Lac-TPE** solution (

To investigate the selectivity of fluorometric detection for CT, as well as the specificity of carbohydrate–protein interactions, **Lac-TPE** was treated with another protein (BSA) under the same conditions, and no significant change of photoluminescence intensity was observed. Furthermore, when **Lac-TPE** was mixed with an equal amount of CTB and BSA in PBS solution, the mixture displayed an intense emission, in which the intensity is almost as same as that observed in the case of CTB (Figure 4).

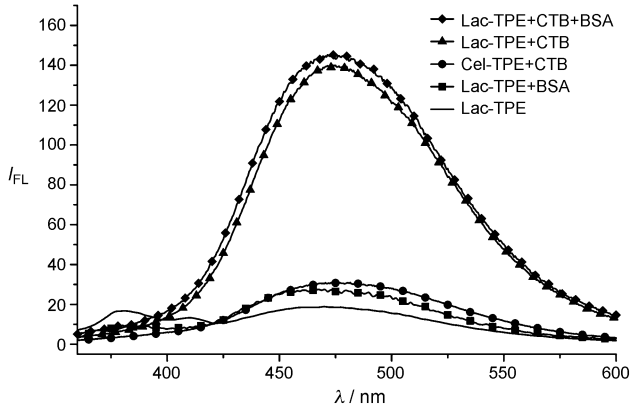



Figure 4. Fluorescence spectra of sugar-TPE derivatives (2.0 μM **Lac-TPE** or **Cel-TPE** in 50 mM PBS solution; pH 7.3) in the absence and presence of different proteins (5.0 μM **CTB** or **BSA** in 50 mM PBS solution; pH 7.3).

As a control study, **Cel-TPE** was also used to investigate the effect of sugar ligand on the selectivity of CT detection. Compared with high fluorescence intensity induced by **Lac-TPE/CTB** ensemble, the CTB cannot "turn on" the fluorescence of **Cel-TPE**, which implies that there is no specific interaction between **Cel-TPE** and CTB. All the results aforementioned substantiate the specificity of interaction between **Lac-TPE** and CTB.

## Conclusions

In summary, neutral lactose-bearing tetraphenylethenes are designed and prepared as "turn-on" luminescent sensors for cholera toxin. Aggregation derived from lactosyl–CTB binding can switch on the fluorescence of water-soluble tetraphenylethylene-based glycoconjugates in PBS solution, which can be used for investigation of carbohydrate–protein interactions based on aggregation-induced emission. We believe that methods inspired by this concept of investigating carbohydrate-mediated biological interactions have promising applications in biomacromolecule detections and glycobiology studies.

## Experimental Section

*Materials and Instrumentation*

All chemical reagents were commercially available and used as received unless otherwise stated. CTB and Bovine Serum Albumin (BSA) were purchased from Sigma–Aldrich Co. Tetrahydrofuran (THF) was purified by distillation from sodium under nitrogen immediately prior to use. Deionized water was obtained with a Millipore purification system (Milli-Q water).

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker DMX400 NMR spectrometer using the solvent peak as internal reference. Mass spectrometry was performed on a Thermo LCQ Deca XP MAX mass spectrometer using the ESI(+) technique. Single-crystal X-ray diffraction analysis of TPE derivative **1** was carried out on a Bruker SMART APEX II CCD diffractometer. Ultraviolet-visible (UV/Vis) spectra were measured on a Perkin–Elmer Lamda 950 UV/Vis spectrometer and fluorescence spectra were measured on a Perkin–Elmer LS 55 luminescence spectrometer using a conventional cell with 1 cm path length.

*Synthesis of TPE derivative **1***

Zn dust (5.31 g, 81.2 mmol) was added to a solution of 4,4′-dimethoxybenzophenone (2.01 g, 8.3 mmol) in 40 mL of dry THF. After refluxing for 20 h, the reaction mixture was cooled to room temperature and filtered. The solvent was evaporated under vacuum and the crude product was purified by column chromatography on silica gel using dichloromethane/petroleum ether (v/v = 1:2) as the eluent. Finally, compound **1** was obtained as a white solid in 90% yield (1.69 g). $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 6.92$ (d, $J = 8.6$ Hz, 8H), 6.63 (d, $J = 8.6$ Hz, 8H), 3.72 ppm (s, 12H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta = 157.9$, 138.5, 137.0, 132.6, 113.1, 55.2 ppm.

*Synthesis of TPE Derivative **2***

Under nitrogen atmosphere, compound **1** (3.00 g, 6.63 mmol) was dissolved and stirred in 40 mL dry dichloromethane. $BBr_3$ (6.0 mL, 63.6 mmol) was quickly added to the solution at −45 °C. After stirring for 30 min, the solution was allowed to warm to room temperature and was stirred for a further 10 h. The reaction mixture was poured into cold water with vigorous stirring until no more precipitate was formed. After filtration and drying, a purple solid **2** was obtained in 93% yield (2.44 g). $^1$H NMR (400 MHz, [$D_6$]DMSO): $\delta = 6.71$ (d, $J = 8.4$ Hz, 8H), 6.49 ppm (d, $J = 8.4$ Hz, 8H). $^{13}$C NMR (100 MHz, [$D_6$]DMSO): $\delta = 155.4$, 137.7, 135.1, 132.0, 114.5 ppm.

*Preparation of TPE Derivative **3***

A mixture of compound **2** (700 mg, 1.77 mmol), propargyl bromide (80 wt% in toluene, 2.0 mL, 18.0 mmol), $K_2CO_3$ (2 g, 14.5 mmol) and $NBu_4Br$ (30 mg) in acetone (30 mL) was refluxed overnight under a nitrogen atmosphere. The mixture was then cooled to room temperature and filtered. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (ethyl acetate/petroleum ether, v/v = 1:5) to give the desired product **3** (794 mg, 82%) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 6.93$ (d, $J = 8.4$ Hz, 8H), 6.70 (d, $J = 8.8$ Hz, 8H), 4.62 (d, $J = 2.4$ Hz, 8H), 2.51 ppm (t, $J = 2.4$ Hz, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta = 156.1$, 138.7, 137.6, 132.6, 114.1, 78.7, 75.6, 55.9 ppm.

*Synthesis of TPE Derivative **5a***

To a suspension of compound **3** (150 mg, 0.27 mmol) and lactose azide derivative **4a** (1.07 g, 1.62 mmol) in THF/$H_2O$ (v/v = 2:1, 30 mL) were added sodium ascorbate (30 mg) and $CuSO_4$ (20 mg) as a catalyst. The mixture was stirred at 70 °C for 6 h and then extracted three times with ethyl acetate (50 mL). The combined organic extracts were concentrated and purified by column chromatography on silica gel (ethyl acetate/petroleum ether, v/v = 4:1) to yield a white solid **5a** (751 mg, 86%). $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 7.78$ (s, 4H), 6.90 (d, $J = 8.4$ Hz, 8H), 6.69 (d, $J = 8.8$ Hz, 8H), 5.85 (d, $J = 8.8$ Hz, 4H), 5.41–5.38 (m, 8H), 5.34 (d, $J = 3.2$ Hz, 4H), 5.09 (s, 8H), 4.96 (dd, $J = 3.2$, 10.4 Hz, 4H), 4.53 (d,

$J$=8.0 Hz, 4H), 4.46 (d, $J$=11.6 Hz, 4H), 4.14–4.05 (m, 16H), 3.96–3.89 (m, 12H), 2.14 (s, 12H), 2.07–2.01 (m, 48H), 1.95 (s, 12H), 1.83 ppm (s, 12H). $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$=170.4, 170.3, 170.2, 170.1, 169.6, 169.2, 169.2, 156.6, 144.9, 138.6, 137.3, 132.6, 121.3, 114.0, 101.2, 85.6, 76.0, 75.7, 72.7, 71.0, 70.9, 70.6, 69.1, 66.7, 61.9, 60.9, 60.5, 21.1, 20.9, 20.7, 20.6, 20.5, 20.4, 20.3 ppm.

*Synthesis of* ***Lac-TPE***

To a solution of compound **5a** (410 mg, 0.13 mmol) in dichloromethane/methanol (v/v=1:3, 20 mL), $CH_3ONa$ (1.0 M in methanol) was added dropwise until the pH value of the solution reached 11. After stirring at room temperature for 5 h, the resultant precipitate was collected by centrifugation at 8000 rpm, washed twice with dichloromethane, dried under vacuum at 40 °C, and finally obtained as a yellowish solid (**Lac-TPE**) in 97% yield (251 mg). $^1$H NMR (400 MHz, [$D_6$]DMSO): $\delta$=8.44 (s, 4H), 6.90 (d, $J$=8.8 Hz, 8H), 6.84 (d, $J$=8.8 Hz, 8H), 5.66 (d, $J$=9.2 Hz, 4H), 5.16–4.57 (m, 36H), 4.26 (d, $J$=7.2 Hz, 4H), 3.87 (t, $J$=9.2 Hz, 4H), 3.78 (d, $J$=10.8 Hz, 4H), 3.67–3.46 (m, 32H), 3.38–3.33 ppm (m, 8H). $^{13}$C NMR (100 MHz, $D_2O$): $\delta$=157.1, 143.9, 139.7, 138.2, 133.3, 124.7, 115.0, 103.9, 88.6, 78.6, 78.3, 76.3, 75.9, 73.6, 73.3, 71.8, 69.5, 61.9, 60.8, 58.2 ppm. MS (ESI-Ion Trap): $m/z$ calcd for $C_{86}H_{112}N_{12}O_{44}$: 2017.86 [$M$]; found: 2075.04 $[M+H_2O+K]^+$.

*Fluorescence Analysis*

A stock solution of **Lac-TPE** was prepared in aqueous phosphate buffer solution (PBS, 50 mM, pH 7.3). Aliquots of **CTB** in the same PBS buffer were added to the solutions of **Lac-TPE**. The final concentration of **Lac-TPE** was 2.0 μM. After each addition, the sample was allowed to equilibrate overnight prior to recording a spectrum. The excitation wavelength was 317 nm and the emission scan ranged from 330–630 nm.

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[16] CCDC 825731 (**1**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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