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Highly Effective Binding of Viologens by *p*-Sulfonatocalixarenes for the Treatment of Viologen Poisoning

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Viologens are showing an increasing number of scientific and technical applications in addition to their use as herbicides. However, their high toxicity poses considerable risks to human health, society, and the environment. In this context, we propose a new therapeutic protocol for the treatment of viologen poisoning. The mechanism of this new protocol is based on host−guest chemistry and involves the effective inhibition of viologen toxicity by the complexation of *p*-sulfonatocalix[*n*]arenes. NMR, ITC, and X-ray crystallography studies indicated that *p*-sulfonatocalix[*n*]arenes could form highly stable complexes with viologens. Electrochemical results showed that the highly effective binding could induce the reduction potentials of viologens to shift to more negative values. Further studies in mice showed that the ingestion of *p*-sulfonatocalix[*n*]arenes significantly decreased the mortality rate of viologen-poisoned mice with lung and liver protection. As a result, *p*-sulfonatocalix[*n*]arenes may have potential application in the clinical treatment of viologen poisoning.

Introduction

Viologens are one class of significant redox couples,[1] widely utilized not only as herbicides[2] but also as probes to study DNA and zeolites,[3] as components of electrochromic display devices,[4] and as prooxidants in stress tests.[5−7] Two typical species of viologens, paraquat (PQ^{2+a}) and diquat (DQ^{2+}), are effective, nonselective, and quick acting herbicides[8] that are used by millions of growers and more than 100 crops in over 120 countries all over the world. Moreover, they are also acutely toxic agrochemicals, and their high toxicity has long posed formidable risks to human health,[9] society, and the environment. Absorption of viologens into the digestive tract, respiratory tract, and skin may result in various diseases or even death.[10−12] PQ^{2+} is also commonly used as a suicide agent in many countries, especially the developing ones. Several governmental organizations, including WHO, US EPA, and ECB et al., have therefore paid great attention to the use of viologens because of their danger to human health, society, and the environment. Some nongovernmental organizations from Asia, America, and Europe even launched the "Stop Paraquat" campaign in 2002. Even so, PQ^{2+} poisonings from either accidental or voluntary ingestion are still frequently reported and result in a high mortality rate owing to: (1) its widespread availability, (2) the low toxic dose, (3) relative cheapness, and (4) few specific antidotes available clinically.[13] To date, as summarized by Dinis-Oliveira et al. in a recent review, "although many treatments have been proposed and attempted empirically based on the pathologic mechanism of toxicity, none are supported by convincing clinical efficacy".[14]

In the present work, we put forward a new therapeutic protocol based on the concept of host−guest chemistry, in which the toxicity of viologens can effectively be inhibited by the host−guest complexation of *p*-sulfonatocalix[*n*]arenes. Calixarenes,[15,16] composed of phenolic units linked via methylene groups, represent the third generation of supramolecular host molecules, next to the crown ethers and cyclodextrins, which are described as "macrocycles with (almost) unlimited possibilities".[17] *p*-Sulfonatocalix[*n*]arenes[18] are one of the most important calixarene derivatives with high water solubility up to 0.1 M.[19] They possess three-dimensional, flexible, π-rich cavities that can selectively bind numerous guest molecules in aqueous solution, including metal ions,[20−22] neutral molecules,[23−25] organic cations,[26−29] and even biological or pharmaceutical molecules.[30−32] More importantly, benefiting from their innocuous nature, *p*-sulfonatocalix[*n*]arenes have exhibited highly diverse biomedical applications, including antiviral and antithrombotic activities, enzyme blocking, and protein complexation.[33−35]

We investigated the binding modes, inclusion abilities, and thermodynamics between *p*-sulfonatocalix[*n*]arenes (*p*-sulfonatocalix[4]arene, C4AS; *p*-sulfonatocalix[5]arene, C5AS; *p*-sulfonatothiacalix[4]arene, TC4AS) and viologens (PQ^{2+} and DQ^{2+}) (Figure 1) by the methods of NMR spectroscopy, X-ray crystallography, and isothermal titration calorimetry (ITC). We found that all the water-soluble calixarenes could form stable inclusion complexes with the two viologen guests at both acidic and neutral conditions. With the toxic mechanism of viologens in hand, the therapeutic effects of *p*-sulfonatocalix[*n*]arenes against viologen poisoning were hypothesized from electrochemical experiments and theoretical analyses and

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[a] Abbreviations: PQ^{2+}, paraquat; DQ^{2+}, diquat; C4AS, *p*-sulfonatocalix[4]arene; C5AS, *p*-sulfonatocalix[5]arene; TC4AS, *p*-sulfonatothiacalix[4]arene; ITC, isothermal titration calorimetry; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; CV, cyclic voltammetry.

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further validated by mice tests. Therefore, the water-soluble *p*-sulfonatocalix[*n*]arenes, as one kind of innocuous and medicinal macrocycles, may have potential applications in the clinical treatment of viologen poisoning.

Results and Discussion

Binding Behavior and Thermodynamics between *p*-Sulfonatocalix[*n*]arenes and Viologens. The binding behavior of *p*-sulfonatocalix[*n*]arenes (C4AS, C5AS, and TC4AS) with viologens (PQ^{2+} and DQ^{2+}) was examined at both acidic and neutral conditions on account of the different biological environments such as serum (pH ca. 7.2) and gastric acid (pH ca. 1.5). All calixarenes, especially C4AS and C5AS, can strongly bind PQ^{2+} and DQ^{2+} at both acidic and neutral conditions, although there are some differences in the complex structures.

NMR spectroscopy is a powerful tool and has been widely used to determine the structures of calixarene complexes by analyzing the complexation-induced shifts.[26] The complex structures of C4AS and C5AS with PQ^{2+} have been demonstrated in our recent report: PQ^{2+} is immersed into the cavity of C4AS in its axial orientation with the methyl group being included first, while it is accumbently included into the cavity of C5AS from the upper rim (Figure 3a and 3b).[36] Herein, to further obtain detailed information about the resulting complex structures of *p*-sulfonatocalix[*n*]arenes with DQ^{2+}, ^{1}H NMR spectra of DQ^{2+} in the presence and absence of calixarenes were measured in pD 2.0 and 7.2 phosphate buffer solutions (Figure 2). All DQ^{2+} protons exhibited a visible upfield shift ($\Delta\delta$) owing to the ring current effect of the aromatic nuclei of calixarenes, which suggests that the DQ^{2+} guest is encapsulated into the calixarene cavities. Moreover, the DQ^{2+} protons are observed as a single resonance because of fast exchange between a free guest and a complexed one on the NMR time scale. The corresponding chemical shift changes ($\Delta\delta$) of DQ^{2+} in the presence of approximately 1 equiv of calixarenes are listed in Table 1. The $\Delta\delta$ values differ from each other, which can be used to deduce the binding geometries of host−guest complexes because the proton with the largest $\Delta\delta$ value would be affected mostly by the ring current effect of the aromatic nuclei of calixarenes. Therefore, according to the sequence of $\Delta\delta$ values, the complex structures are determined such that DQ^{2+} is immersed into the cavities of calixarenes in the acclivitous orientation (Figure 3c). The acclivitous degrees are distinguishable for the three hosts and are in the order of the cavity sizes: C4AS < TC4AS < C5AS. This result is consistent with the complex structures of *p*-sulfonatocalix[*n*]arenes with 2,2′-dipyridinium in our previous work.[29] Furthermore, the deduced complex structures were validated

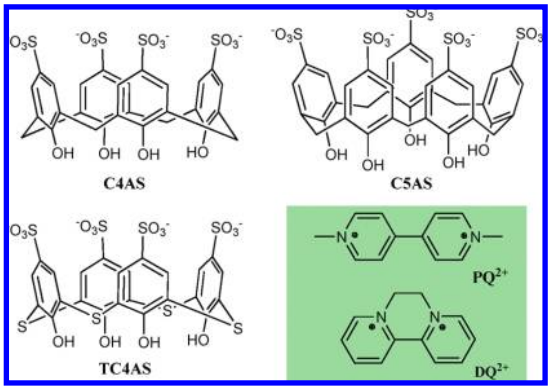

Figure 1. Structural illustration of *p*-sulfonatocalix[*n*]arenes (C4AS, C5AS, and TC4AS) and viologens (PQ^{2+} and DQ^{2+}).

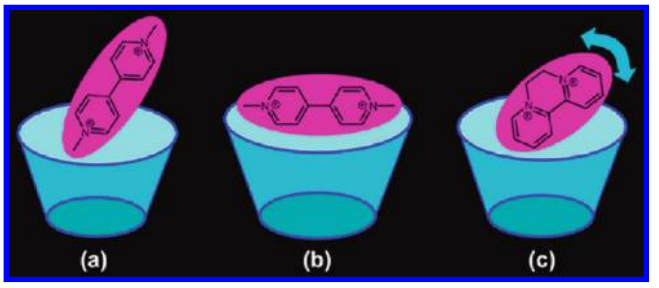

Figure 3. The deduced binding manners of PQ^{2+} with C4AS (a) and C5AS (b), and DQ^{2+} with *p*-sulfonatocalix[*n*]arenes (c) according to NMR spectra.

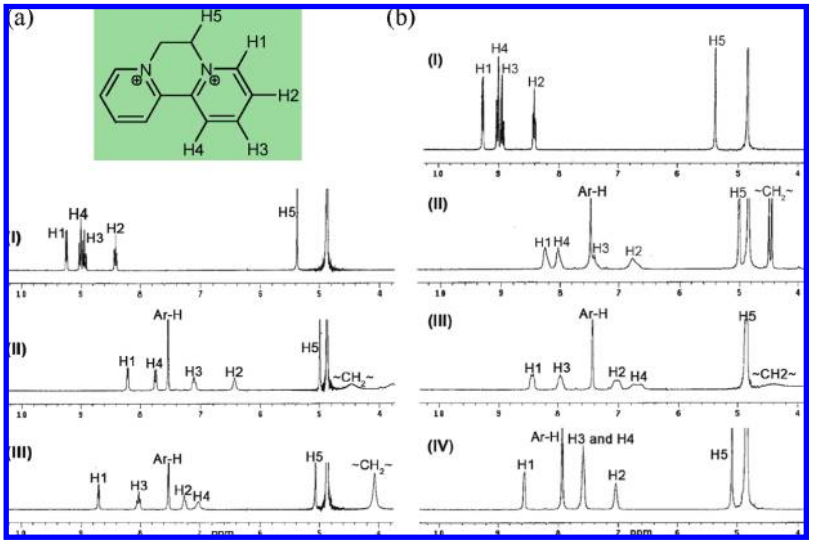

Figure 2. ^{1}H NMR spectra of DQ^{2+} in the absence (and presence) of C4AS, C5AS, or TC4AS: (a) pD = 2.0; (b) pD = 7.2. The ^{1}H NMR spectrum of the complex of TC4AS with DQ^{2+} under acidic conditions was not measured owing to the poor water-solubility. ((I) DQ^{2+}; (II) C4AS+DQ^{2+}; (III) C5AS+DQ^{2+}; (IV) TC4AS+DQ^{2+}).

by the crystal structures of C4AS and TC4AS upon complexation with DQ^{2+} (Figure 4).

Quantitative data for the host−guest complexation obtained from ITC experiments, including complex stability constants (K_S), enthalpy changes ($\Delta H°$) and entropy changes ($\Delta S°$), are listed in Table 2. In all cases, the titration data can be fit well by computer simulation using the "one set of binding sites" model and repeated as 1:1 complex formation, so that the higher-order complexes did not need to be postulated. Under both acidic and neutral conditions, all three *p*-sulfonatocalix[*n*]arene hosts can form stable inclusion complexes with PQ^{2+} and DQ^{2+} guests, and all intermolecular complexation is driven mainly by favorable enthalpy changes, accompanied by small negative or positive entropy changes. This indicates that π-stacking, hydrogen bonding, and van der Waals interactions may play a pivotal role in the host−guest complexation.[37]

Table 1. Chemical Shift Changes ($\Delta\delta$, ppm) of DQ^{2+} in the Presence of C4AS, C5AS, or TC4AS (pD 2.0 and 7.2)[a]

pD	host	H1	H2	H3	H4	H5
2.0	C4AS	−1.05	−1.97	−1.83	−1.27	−0.38
	C5AS	−0.54	−1.15	−0.91	−1.99	−0.29
	TC4AS			[b]		
7.2	C4AS	−1.00	−1.64	−1.52	−0.99	−0.36
	C5AS	−0.83	−1.38	−1.00	−2.33	−0.55
	TC4AS	−0.71	−1.37	−1.35	−1.43	−0.29

[a] $\Delta\delta = \delta$(presence of 1 equiv of host) − δ(free guest). Negative values indicate upfield shift. [b] The ^{1}H NMR spectrum was not measured owing to the poor water solubility of the complex of TC4AS with DQ^{2+} under acidic conditions.

The K_S values of TC4AS are over 1 order of magnitude smaller than those of C4AS owing to the replacement of the methylene linkages of calix[4]arenes by sulfide. However, there are two different thermodynamic origins that cause the binding difference between C4AS and TC4AS. Under acidic conditions, the weaker binding abilities of TC4AS are mainly reflected by the enthalpy term and the lower π-electron density of the cavity leads to less effective π-stacking interactions with guests. Under neutral conditions, the weaker binding abilities of TC4AS originate mainly from the more unfavorable entropy changes. This observation contrasts with previous results showing that TC4AS often presents more favorable entropy changes than C4AS owing to its better flexibility of framework.[25,29,38] It also indicates that the cavity size and preorganized structure of TC4AS do not fit well with PQ^{2+} and DQ^{2+} guests, leading to a greater loss of conformational degrees of freedom and structure freezing upon complexation. For the C4AS and C5AS pairs, C4AS provides stronger binding ability under acidic conditions because its smaller cavity and relatively higher π-electron density can bind the guests much tighter than C5AS, leading to a more favorable π-stacking interaction. On the contrary, C5AS provides stronger binding ability due to the suitable induced-fit interaction between C5AS and guests under neutral conditions.[36]

Upon complexation with the same host, the K_S values of DQ^{2+} are always larger than those of PQ^{2+} under both acidic and neutral conditions, an observation which is supported by not only the enthalpy but also by the entropy terms. It indicates that the position of the nitrogen atoms in the viologens exerts a certain influence on the complex stabilities. In comparison with PQ^{2+}, DQ^{2+} is more prone to form π-stacking interactions with calixarene hosts and then shows

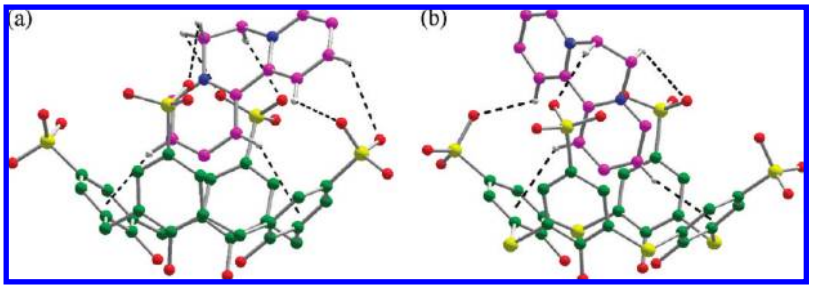

Figure 4. Crystal structures of the complexes of C4AS (a) and TC4AS (b) with DQ^{2+} in pH 1−2 H_2O-HCl aqueous solutions. As shown in this figure, DQ^{2+} is included into the cavity of C4AS and TC4AS in the acclivitous orientation.

Table 2. Complex Stability Constants (K_S/M^{-1}), Enthalpy ($\Delta H°/(kJ\cdot mol^{-1})$), and Entropy ($T\Delta S°/(kJ\cdot mol^{-1})$) Changes for 1:1 Intermolecular Complexation of *p*-Sulfonatocalix[*n*]arenes with PQ^{2+} and DQ^{2+} in Phosphate Buffer Solutions (pH 2.0 and 7.2) at 298.15 K

conditions, pH	complexes	K_S	$\Delta H°$	$T\Delta S°$
2.0	C4AS + PQ^{2+}[a]	3.09×10^4	−28.18	−2.53
	C5AS + PQ^{2+}[a]	5.50×10^3	−20.58	0.79
	TC4AS + PQ^{2+}	$(2.22 \pm 0.01) \times 10^3$	−21.58 ± 0.05	−2.47 ± 0.05
	C4AS + DQ^{2+}	$(5.40 \pm 0.01) \times 10^5$	−34.42 ± 0.01	−1.69 ± 0.02
	C5AS + DQ^{2+}	$(1.12 \pm 0.04) \times 10^5$	−32.11 ± 0.01	−3.29 ± 0.07
	TC4AS + DQ^{2+}	$(2.39 \pm 0.06) \times 10^4$	−27.50 ± 0.22	−2.49 ± 0.28
7.2	C4AS + PQ^{2+}[a]	9.33×10^4	−31.98	−3.62
	C5AS + PQ^{2+}[a]	2.51×10^5	−31.52	−0.67
	TC4AS + PQ^{2+}	$(8.68 \pm 0.07) \times 10^3$	−31.01 ± 0.06	−8.53 ± 0.07
	C4AS + DQ^{2+}	$(7.95 \pm 0.16) \times 10^5$	−33.90 ± 0.13	−0.21 ± 0.08
	C5AS + DQ^{2+}	$(3.23 \pm 0.06) \times 10^6$	−32.78 ± 0.04	4.39 ± 0.01
	TC4AS + DQ^{2+}	$(4.57 \pm 0.13) \times 10^4$	−32.26 ± 0.03	−5.65 ± 0.04

[a] Reference 36.

the more favorable enthalpy changes. Moreover, the entropy losses upon complexation with DQ^{2+} are obviously less than those of PQ^{2+} at pH 7.2. Consequently, the complexation with DQ^{2+} gives rise to a smaller conformational loss of calixarenes.

Potential Therapeutic Mechanism of *p*-Sulfonatocalix[*n*]arenes for Viologen Poisoning. PQ^{2+} and DQ^{2+} are two of the most commonly used herbicides that belong to the chemical family of bipyridylium quaternary ammonium herbicides. They have similar chemical and physical properties and possess a similar mode of action and toxicodynamic mechanism based on the redox cycling.[39] The biochemical mechanism of PQ^{2+} toxicity can be found in Figure 5.[14] Upon entry into the cell, PQ^{2+} is enzymatically reduced to form the radical cation $PQ^{\bullet+}$. $PQ^{\bullet+}$ then rapidly reduces O_2 to form $O_2^{\bullet-}$. The redox reaction between $PQ^{\bullet+}$ and O_2 is very fast, with a rate constant of 7.7×10^8 $M^{-1} \cdot s^{-1}$.[40] This will lead to the production of other reactive oxygen species, H_2O_2 and $HO^{\bullet}$. H_2O_2 can further generate $HO^{\bullet}$ via the Fenton reaction. $HO^{\bullet}$ is the consequent toxic radical with deleterious cellular effects by oxidizing lipids, proteins, and nucleic acids. In summary, the biochemical mechanism of PQ^{2+} toxicity involves the elevation of intracellular levels of superoxide ($O_2^{\bullet-}$) by redox cycling.

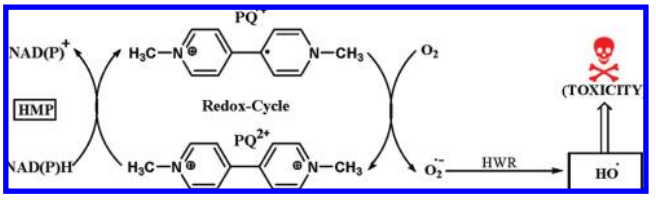

Figure 5. Biochemical mechanism of PQ^{2+} toxicity (HWR: Haber−Weiss reaction; HMP: hexose monophosphate pathway).

With the toxic mechanism of PQ^{2+} in hand, the biochemical mechanism of treatment for viologen poisoning by *p*-sulfonatocalix[*n*]arenes can be predicted from four viewpoints. First, PQ^{2+} and DQ^{2+} could be tightly included into the cavity of *p*-sulfonatocalix[*n*]arenes and thus would have less opportunity to interact with the reducing agents in the cell. Second, the first reduction potentials of both PQ^{2+} and DQ^{2+} are pronouncedly shifted to more negative values upon complexation with *p*-sulfonatocalix[*n*]arenes (Figure 6a,b), which makes generation of their radical cations more difficult, and then decreases the production of the toxic $HO^{\bullet}$. This phenomenon can also be observed by the naked eye as well as by electrochemical measurements. The PQ^{2+} and DQ^{2+} solutions changed from colorless to yellow upon addition of *p*-sulfonatocalix[*n*]arenes (Figure 6c,d), suggesting that the charge-transfer complexes were formed between them. Third, the resulting radicals can be deactivated by hydrogen atom abstraction from the active phenolic hydroxyls at the lower-rims of *p*-sulfonatocalix[*n*]arenes. Fourth, the generation of $HO^{\bullet}$ may be catalyzed by traces of transition metal ions.[41,42] *p*-Sulfonatocalix[*n*]arenes have the capability to bind transition metal ions,[43] which could possibly inhibit their catalytic effect.

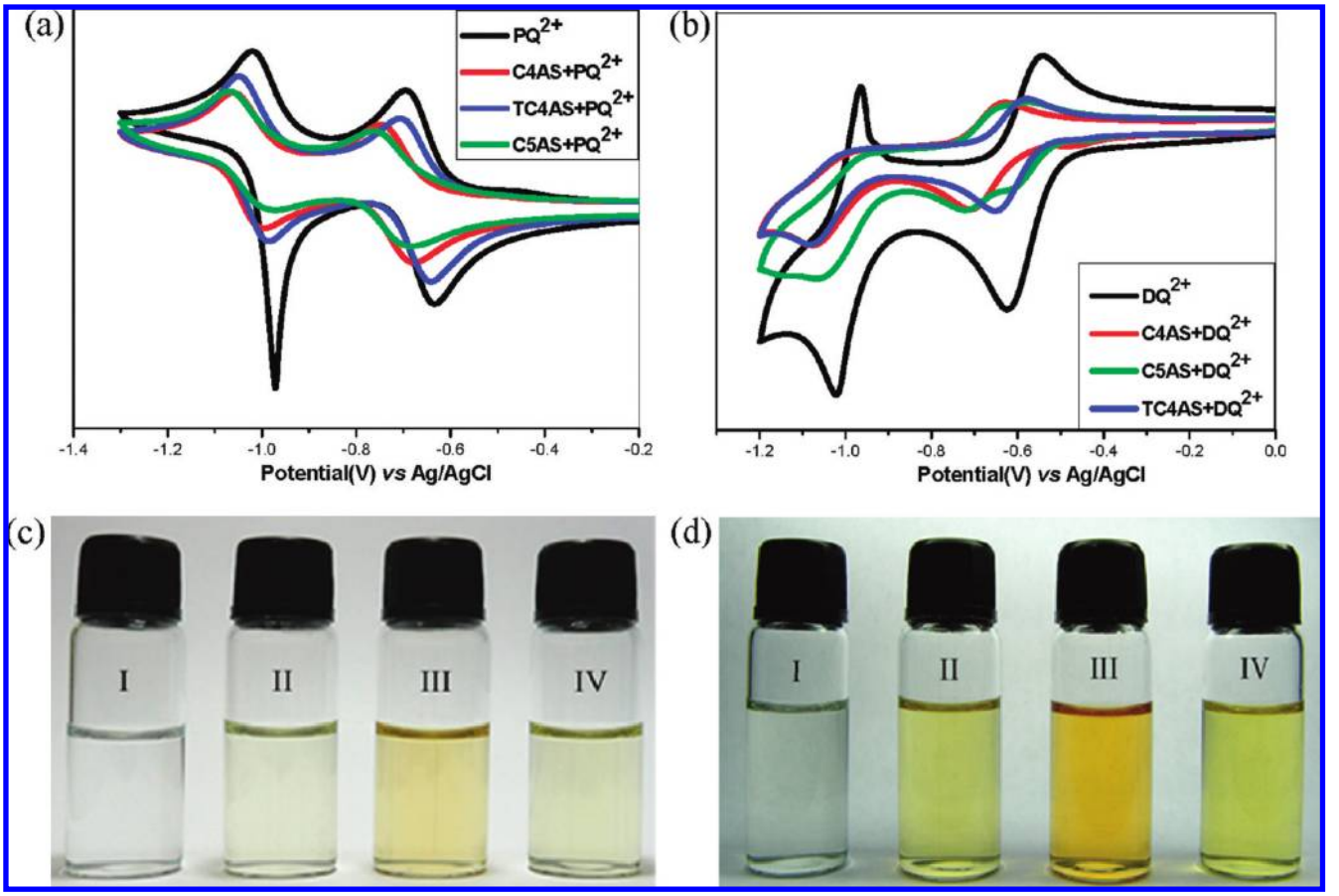

Figure 6. Cyclic voltammetric curves of PQ^{2+} (a) and DQ^{2+} (b) (1.0 mM in pH 7.2 phosphate buffer solution) in the absence and presence of 1 equiv of C4AS, C5AS, and TC4AS. The scan rate is 100 mV/s. Pictures showing the color changes of PQ^{2+} (c) and DQ^{2+} (d) upon complexation with 1 equiv of *p*-sulfonatocalix[*n*]arenes (1.0 mM in pH 7.2 phosphate buffer solution), (I) free viologens, (II) +C4AS, (III) +C5AS, and (IV) +TC4AS.

Therapeutic Effects of *p*-Sulfonatocalix[*n*]arenes for Viologen Poisoning Validated by Mice Tests. We further performed tests in mice to examine the therapeutic effects of *p*-sulfonatocalix[*n*]arenes for viologen poisoning. Herein, C5AS was employed as the therapeutic reagent for the following reasons: (1) C5AS can form highly stable complexes with viologens under both acidic and neutral conditions, (2) C5AS can induce the largest negative shifts of reduction potentials of viologens among the three *p*-sulfonatocalix[*n*]arenes, accompanied by the most prominent charge-transfer phenomenon, (3) C5AS possesses more active phenolic hydroxyls at its lower-rim than C4AS and TC4AS. Moreover, the larger analogue, *p*-sulfonatocalix-[6]arene, was also excluded because of its uncertain conformation and relatively weak binding ability.[44] C5AS is therefore expected to have better therapeutic capability for viologen poisoning than the other calixarenes. The viologen we selected to perform the mice tests was PQ^{2+} because DQ^{2+} poisonings are not as common as PQ^{2+} poisonings.

First, we employed 40 mice, which were divided into four groups stochastically. The first control group was administered with normal saline (0.9% w/v NaCl; 100 μL/20 g of body weight). The second group was administered with a solution of C5AS alone (9.7% w/v C5AS; 100 μL/20 g of body weight). The third group was administered with a solution of PQ^{2+} (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight) according to the published LD_{50} value in mice (120 mg/kg of body weight; per os).[45] The fourth group was administered with a solution of the C5AS·PQ^{2+} complex (12.1% w/v C5AS·PQ^{2+} complex; 100 μL/20 g of body weight). We watched these experimental mice for 13 days following the administrations and obtained the mortality rate for each group (Figure 7a). On day 14, the living mice were then sacrificed for weighing (see Figure S1a, Supporting Information), followed by sampling the liver and lung tissues for pathological examination. All mice in group 2 remained alive for the duration of the experiment, and the average weight of group 2 (33.4 ± 2.9 g) was statistically equivalent to that of group 1 (32.8 ± 3.8 g) ($P > 0.05$), which indicates that C5AS is innocuous to the mice in accordance with the previously published results for *p*-sulfonatocalix[*n*]arenes.[33] Recently, the first in vivo studies of the biodistribution and pharmacokinetics of C4AS with regard to mice by the method of radioisotopic labeling have been described by Coleman et al.,[46] which also shows that there is no acute toxicity for doses up to 100 mg/kg and the molecule is rapidly cleared via elimination in urine. There is no significant uptake in the organs, and particularly in the liver and spleen. The mortality rate of group 3 was 90%, and the weight of the surviving mouse was only 14.0 g, which clearly demonstrates the serious toxicity of PQ^{2+} to the mice. The mortality rate of group 4 was decreased to 20%. Moreover, in stark contrast to group 3, the average weight of group 4 (30.8 ± 5.7 g) was statistically equivalent to those of groups 1 and 2 ($P > 0.05$). All of these data indicate that the complexation of C5AS can markedly inhibit the fatal toxicity of PQ^{2+}. The gross changes of lung and liver were observed by the naked eye (see Figure S1b and S2, Supporting Information). The tissues were fixed and sectioned for microscopic examination of the structure (Figure 7b,c). No appreciable pathological changes were observed in the lungs and livers of mice belonging not only to groups 1 and 2, but also those in group 4. In contrast, the microscopic examination of lungs and livers of the surviving mouse in group 3 showed a massive destruction of structures of the tissues.

To further investigate the feasibility of treating PQ^{2+} poisoning by C5AS, we performed tests on another four groups of mice with different intervals of C5AS administration after PQ^{2+} poisoning. All four groups of mice were poisoned by ingesting the PQ^{2+} solution (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight), then administered with a solution of C5AS (19.4% w/v C5AS; 100 μL/20 g of body weight) immediately, half an hour later, one hour later, and two hours later, respectively (groups 5−8). Taking the estimated average arrival time of the patient to the hospital after PQ^{2+} poisoning into account, the longest interval we chose is two hours. We also observed these experimental mice for 13 days following the administrations, and obtained the mortality rates for groups 5−8 (Figure 8a). The living mice on day 14 were dealt with in the same manner as groups 1−4. The mortality rates of groups 5, 6, and 8 are the same at 40%, while it was surprising to observe a zero mortality rate for group 7. The total mortality rate of groups 5−8 is therefore 30%, which is still far lower than that of group 3. The average weights of mice in groups 5−8 were in the range of 31.0−33.0 g (see Figure S3a, Supporting Information), and there were no appreciable pathological changes in lung and liver observed either by the naked eye (see Figure S3b and S4, Supporting Information) or by microscope (Figure 8b,c). The present results from the mice in groups 5−8 indicate that C5AS shows a potential therapeutic capability, even if the mice were treated two hours after PQ^{2+} poisoning.

To explain the extraordinarily high survival rate for group 7, we further studied the time-course of PQ^{2+} relative concentration in mice plasma after an oral administration of PQ^{2+} (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight) and C5AS·PQ^{2+} complex (12.1% w/v C5AS·PQ^{2+} complex; 100 μL/20 g of body weight) respectively. As shown in Figure 9, only one peak is observed in the case of both PQ^{2+} and C5AS·PQ^{2+} complex with similar peak area and distinguishable peak times, about 90 min for PQ^{2+} and 15 min for C5AS·PQ^{2+} complex. The time difference clearly indicates that PQ^{2+} exists in the form of complex in vivo under our mice test conditions. The peak time of C5AS·PQ^{2+} complex is somewhat comparable to that the C4AS amount present in blood peaks after 30 min at a dose of 40 mg/kg of mice body weight.[46] This indicates that the biodistribution of *p*-sulfonatocalixarene in blood is about 1 h faster than that of PQ^{2+}, which reasonably explains us why the therapeutic effect of group 7 is better than the other groups 5, 6, and 8.

In an excellent work, Dinis-Oliveira et al. reported the treatment of sodium salicylate and its prodrug for viologen poisoning in recent years, which exhibits the best healing effect up to now.[47−50] The mortality rate of viologen-poisoned rats changed from 100% (25 mg/kg of body weight) and 60% (125 mg/kg of body weight) for PQ^{2+} group to zero for treatment by sodium salicylate and its prodrug (200 mg/kg of body weight, ip), respectively, two hours after PQ^{2+} poisoning with a protection of PQ^{2+}-induced apoptosis in the rat lung. The presence of active hydroxyl in sodium salicylate and its capability of complexation with PQ^{2+} play an important role in the treatment of viologen poisoning. It is coincident that we also designed the therapeutic strategies based on the concept of inclusion/complexation. They did not report the complexation stability constant of PQ^{2+} with

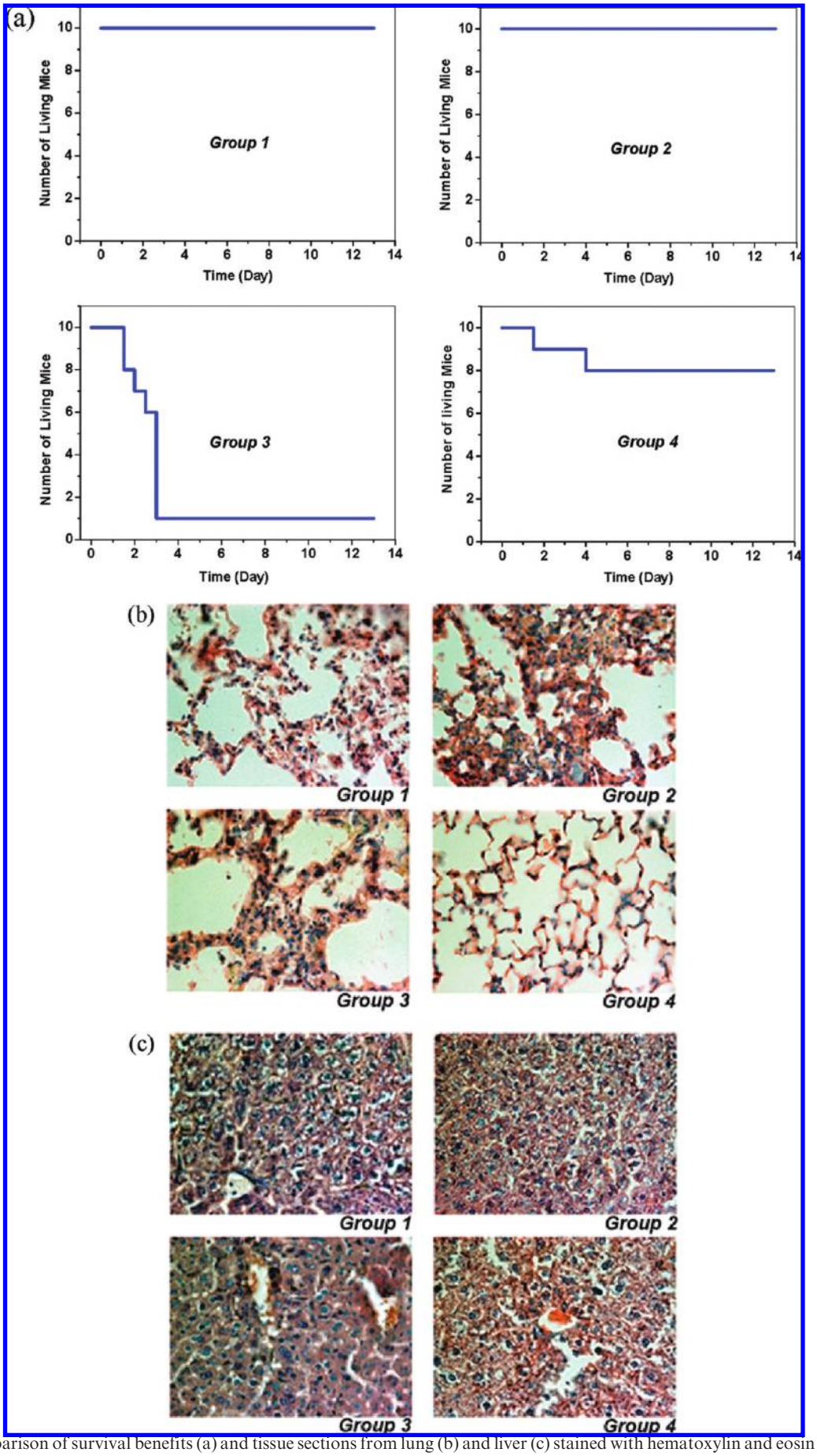

Figure 7. Comparison of survival benefits (a) and tissue sections from lung (b) and liver (c) stained with hematoxylin and eosin (HE, ×400) for groups 1−4.

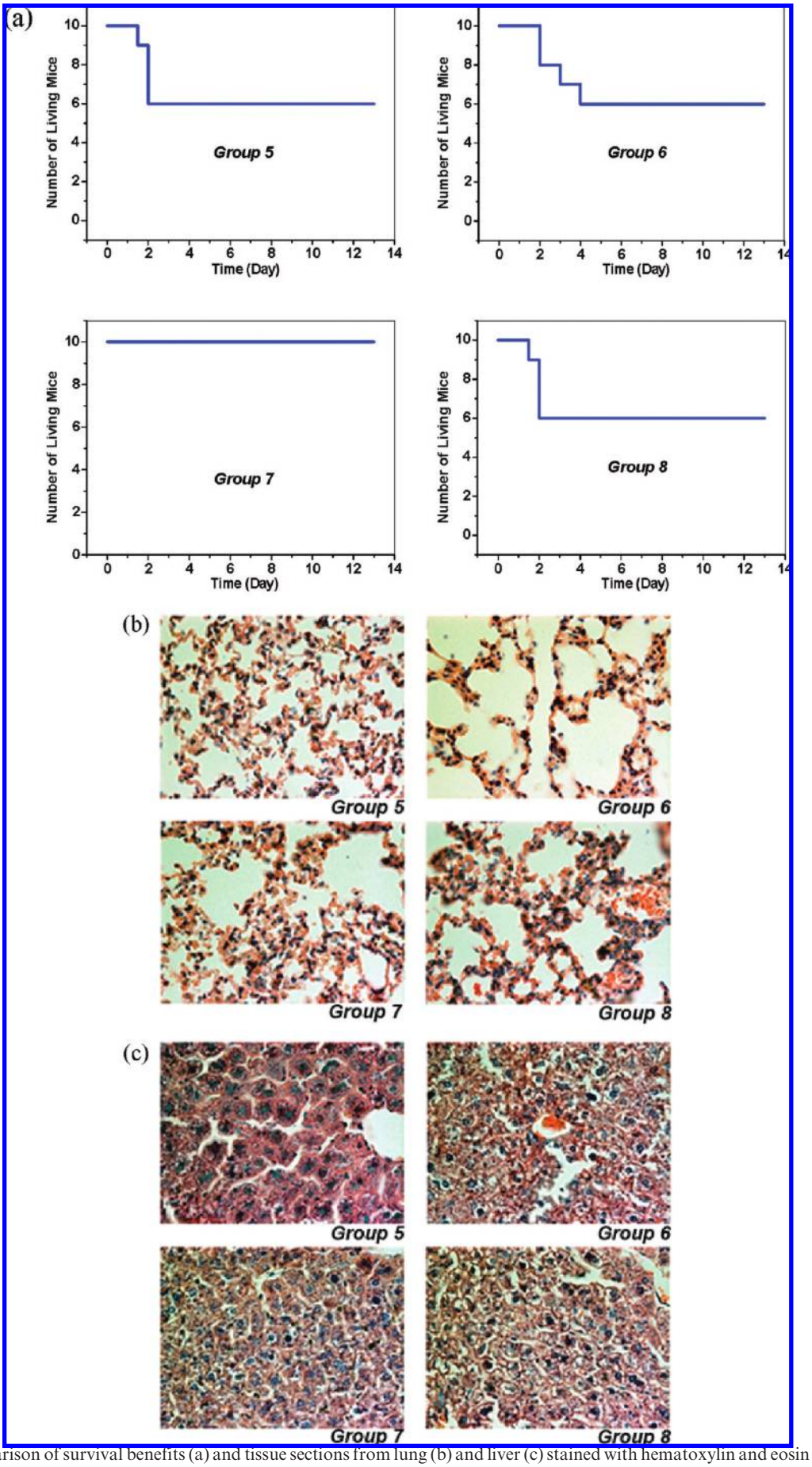

Figure 8. Comparison of survival benefits (a) and tissue sections from lung (b) and liver (c) stained with hematoxylin and eosin (HE, × 400) for groups 5−8.

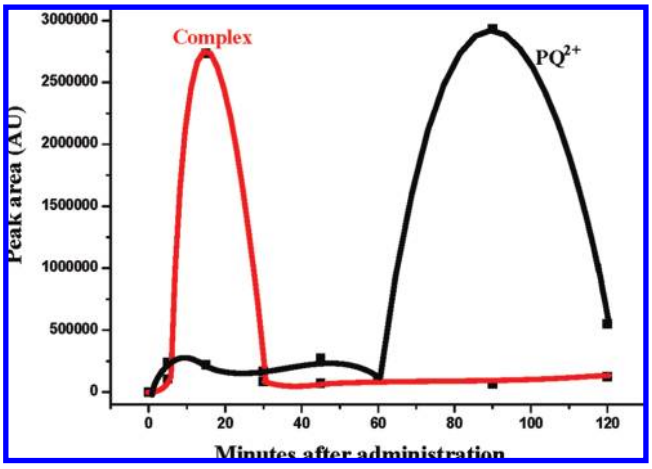

Figure 9. Time-course of PQ^{2+} relative concentration in mice plasma after an oral administration of PQ^{2+} (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight) and C5AS·PQ^{2+} complex (12.1% w/v C5AS·PQ^{2+} complex; 100 μL/20 g of body weight), respectively.

sodium salicylate, and then we can not compare its binding stability with that of C5AS·PQ^{2+} complex here. On the basis of the preliminary results in present study, C5AS maybe act as a more promising healing reagent on account of its polyhydroxy ring structure and strong binding ability to PQ^{2+}. It is worth mentioning that we only treated the poisoned mice by administering C5AS once through oral ingestion in the present study; for future clinical applications, the therapeutic effects of *p*-sulfonatocalix[*n*]arenes for viologen poisoning could be improved by modifying the methods of treatment, such as intraperitoneal injection (ip), intravenous injection (iv), continuous infusion, or repeated administration. In addition, therapy by *p*-sulfonatocalix[*n*]arenes can be positively enhanced by combining with other therapeutic methods, including (1) preventing viologen absorption, (2) increasing viologen elimination, (3) supportive therapies, and (4) preventing lung damage.[14]

Conclusion

In summary, we have explored the potential application of *p*-sulfonatocalix[*n*]arenes to treat viologen poisoning in this study. On the basis of the biochemical mechanism of viologen toxicity, *p*-sulfonatocalix[*n*]arenes were inferred to be capable of inhibiting the viologen toxicity because of their four characteristics, i.e., high binding abilities to viologens, inducing negative shifts in the reduction potentials of viologens, hydrogen transfer to (toxic) radicals, and coordination of transition metal ions. The therapeutic effect was actually validated by mice tests. The mortality rate was significantly decreased when the viologen-poisoned mice were administered with *p*-sulfonatocalix[*n*]arenes, and the ingestion of *p*-sulfonatocalix[*n*]arenes can also effectively prevent PQ^{2+}-induced destruction of structures of the tissues. The present results pave a new way for the effective treatment of viologen poisoning from the viewpoint of host−guest chemistry. Furthermore, *p*-sulfonatocalix[*n*]arenes show considerable promise as novel medicines in the effective clinical treatment of viologen poisoning.

Experimental Section

Materials. The three host compounds, *p*-sulfonatocalix[4]arene tetrasodium (C4AS),[51] *p*-sulfonatocalix[5]arene pentasodium (C5AS),[52] and *p*-sulfonatothiacalix[4]arene tetrasodium (TC4AS),[53] and two guests, PQ^{2+}[36] and DQ^{2+},[54] were synthesized and purified according to previously reported procedures. These compounds were identified by ^{1}H NMR spectrum on a Varian 300 spectrometer and elemental analysis on a Perkin-Elmer-2400C instrument. The found elemental analysis values for all the prepared compounds are within ±0.4% of the calculated values, confirming their ≥95% purity. The phosphate buffer solution of pH 2.0 was prepared by dissolving sodium dihydrogen phosphate in distilled, deionized water to make a 0.1 M solution, which was then adjusted to pH 2.0 by phosphoric acid. The phosphate buffer solution of pH 7.2 was prepared by dissolving disodium hydrogen phosphate ($Na_2HPO_4·12H_2O$, 25.79 g) and sodium dihydrogen phosphate ($NaH_2PO_4·2H_2O$, 4.37 g) in distilled, deionized water (1000 mL) to make a 0.1 M solution. The phosphate D_2O buffer solution of pD 2.0 was prepared by dissolving sodium dihydrogen phosphate (NaH_2PO_4, 0.2379 g) in 20.00 mL D_2O to obtain a 0.1 M solution and then was adjusted to pD 2.0 by DCl. The phosphate D_2O buffer solution of pD 7.2 was prepared by dissolving sodium dihydrogen phosphate (NaH_2PO_4, 0.0672 g) and disodium hydrogen phosphate (Na_2HPO_4, 0.2045 g) in 20.00 mL D_2O to make a 0.1 M solution. The pH and pD values of buffer solutions were verified on a Sartorius pp-20 pH meter calibrated with two standard buffer solutions. pH readings were converted to pD by adding 0.4 units.

Instruments. ^{1}H NMR spectra were recorded at pD 2.0 and 7.2 on a Varian Mercury VX300 spectrometer using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an external reference. The host and guest were mixed in a 1:1 stoichiometry.

A thermostatted and fully computer-operated isothermal calorimetry (VP-ITC) instrument, purchased from Microcal Inc., Northampton, MA, was used for all microcalorimetric experiments. The VP-ITC instrument was calibrated chemically by measurement of the complexation reaction of β-cyclodextrin with cyclohexanol, and the obtained thermodynamic data were in good agreement (error <2%) with the literature data. All microcalorimetric titrations between *p*-sulfonatocalix[*n*]arenes and viologens were performed in aqueous phosphate buffer solution (pH 2.0 or pH 7.2) at atmospheric pressure and 298.15 K. Each solution was degassed and thermostatted by a ThermoVac accessory before the titration experiment. Twenty-five successive injections were made for each titration experiment. A constant volume (10 μL/injection) of guest (or host) solution in a 0.250 mL syringe was injected into the reaction cell (1.4227 mL) charged with host (or guest) molecule solution in the same buffer solution. A representative titration curve was shown in Figure 10. As can be seen from Figure 10, each titration of DQ^{2+} into the sample cell gave an apparent reaction heat caused by the formation of inclusion complex between C5AS and DQ^{2+}. The reaction heat decreases after each injection of DQ^{2+} because less and less host molecules are available to form inclusion complexes. A control experiment was carried out in each run to determine the dilution heat by injecting a guest (or host) buffer solution into a pure buffer solution containing no host (or guest) molecules. The dilution heat determined in these control experiments was subtracted from the apparent reaction heat measured in the titration experiments to give the net reaction heat.

The net reaction heat in each run was analyzed by using "one set of binding sites" model (ORIGIN software, Microcal Inc.) to simultaneously compute the binding stoichiometry (N), complex stability constant (K_S), standard molar reaction enthalpy ($\Delta H°$), and standard deviation from the titration curve. Generally, the first point of titration curve was removed, considering that the concentration of host in the cell far exceeded the concentration of the guest. Knowledge of the complex stability constant (K_S) and molar reaction enthalpy ($\Delta H°$) enabled calculation of the standard free energy ($\Delta G°$) and entropy changes ($\Delta S°$) according to

$$\Delta G° = -RT \ln K_S = \Delta H° - T\Delta S°$$

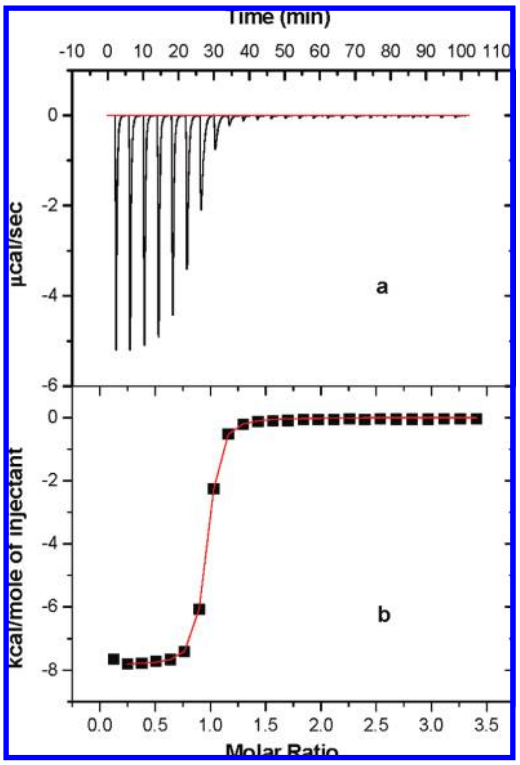

Figure 10. Microcalorimetric titration of C5AS with DQ^{2+} in phosphate buffer solution (pH = 7.2) at 298.15 K. (a) Raw data for sequential 25 injections (10 μL per injection) of DQ^{2+} solution (1.96 mM) injecting into C5AS solution (0.11 mM). (b) Apparent reaction heat obtained from the integration of calorimetric traces.

where R is the gas constant and T is the absolute temperature.

A typical curve fitting result for the complexation of DQ^{2+} with C5AS at pH 7.2 was shown in Figure 11. To check the accuracy of the observed thermodynamic parameters, two independent titration experiments were carried out to afford self-consistent thermodynamic parameters, and their average values were listed in Table 2.

The cyclic voltammetry (CV) measurements were carried out on a BAS Epsilon electrochemical analyzer with C3 cell stand. All the solutions were prepared in pH 7.2 phosphate buffers (0.1 M) at 25 °C, and deoxygenated by purging with dry nitrogen for at least 15 min before each experiment. The glassy carbon working electrode was polished with 0.05 μm BAS alumina suspension on a brown Texmet polishing pad, sonicated in distilled water for a few minutes to remove any residual alumina particles, and then rinsed with ethanol before use. A platinum wire was used as the counter electrode. The measured potentials were recorded with respect to an Ag/AgCl (immersed in a solution containing 3 M sodium chloride) reference electrode.

Mice Tests. Eighty male Kun-Ming mice with weights of 19.5 ± 0.5 g were obtained from the Laboratory Animal Center, Academy of Military Medical Sciences, CPLA (People's Liberation Army, China). PQ^{2+} solution (20% w/v) with vomit used as herbicide was purchased from the market. We divided 80 mice into 8 groups stochastically and each group had 10 mice. Each group was individually housed during the experimental period in a polypropylene cage with a stainless-steel net at the top and wood chips at the screen bottom. Fresh tap water was given to each group ad libitum during the entire experiment. All the experimental mice were fasted for 24 h before experiment. The administrations of normal saline (0.9% w/v NaCl), PQ^{2+}, C5AS, and C5AS·PQ^{2+} complex were all made per os in an injection volume of 100 μL/20 g of body weight. Group 1 was administered with normal saline (100 μL/20 g of body weight) as

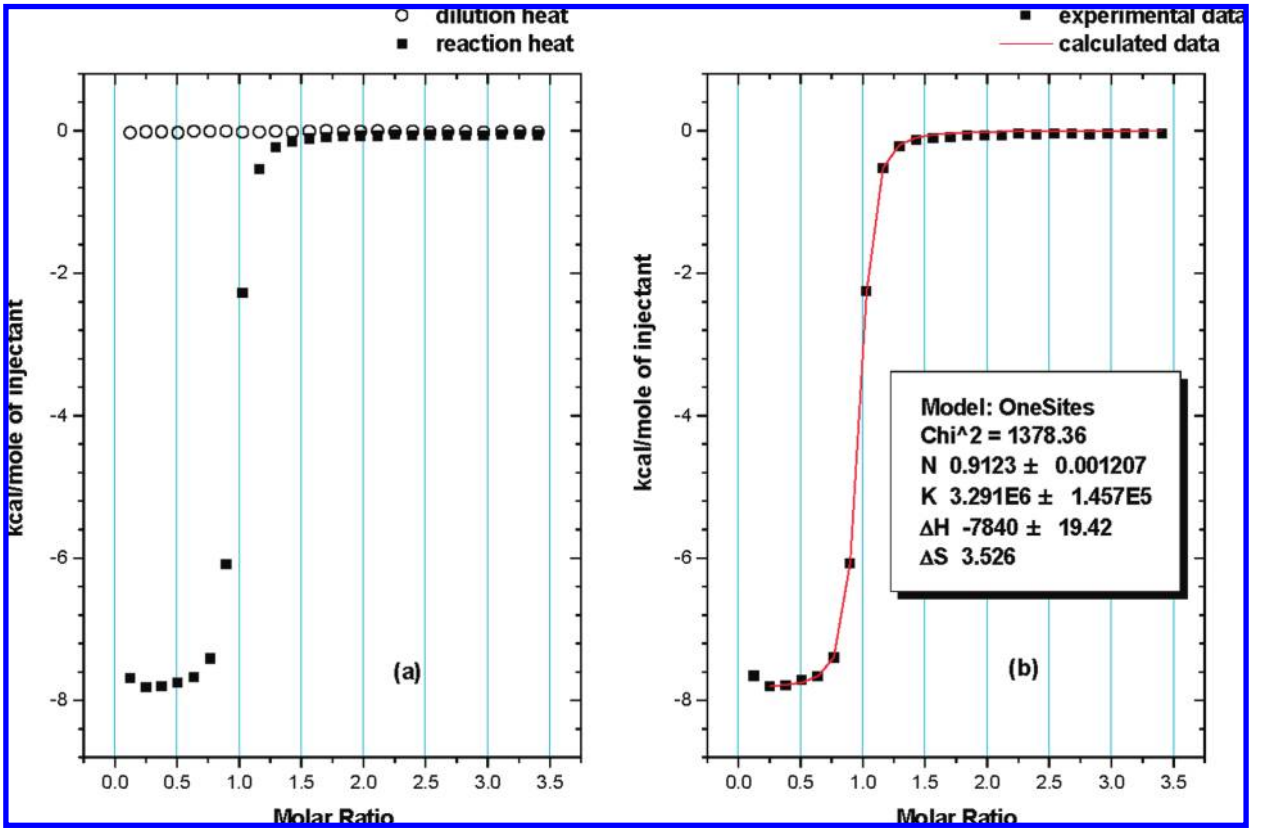

Figure 11. (a) Heat effects of the dilution and of the complexation reaction of DQ^{2+} with C5AS for each injection during titration microcalorimetric experiment. (b) "Net" heat effects of complexation of DQ^{2+} with C5AS for each injection, obtained by subtracting the dilution heat from the reaction heat, which was fitted by computer simulation using the "one set of binding sites" model.

a control experiment. Group 2 was administered with a solution of C5AS (9.7% w/v C5AS; 100 μL/20 g of body weight) to determine the toxicity of C5AS. Group 3 was administered with a solution of PQ^{2+} (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight) according to the published LD_{50} value in mice (120 mg/kg of body weight; per os). Group 4 was administered with a solution of the C5AS·PQ^{2+} complex (12.1% w/v C5AS·PQ^{2+} complex; 100 μL/20 g of body weight) to observe if the toxicity of PQ^{2+} could be inhibited upon complexation with C5AS. Groups 5−8 were poisoned by ingesting the PQ^{2+} solution (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight), then administered with a solution of C5AS (19.4% w/v C5AS; 100 μL/20 g of body weight) immediately, half an hour later, one hour later, and two hours later, respectively. We watched these experimental mice for 13 days following the administrations and obtained the mortality rate for each group. On day 14, the living mice were then sacrificed for weighing followed by dissection to observe the gross changes in lung and liver through the naked eye. After that, the tissues were fixed and sectioned for microscopic examination of the structure. All the pathological sections were photographed by LEICA CTRMIC photograph system. All experimental procedures were approved and in accordance with China's National Code of Animal Care for Scientific Experimentation. The weight of the mice is expressed as the mean ± SD and *t* test was used for statistical analysis of the data.

Another 48 male Kun-Ming mice with weights of 19.5 ± 0.5 g were employed in the following mice tests to get the time-course of PQ^{2+} relative concentration in mice plasma after an oral administration of PQ^{2+} and C5AS·PQ^{2+} complex, respectively, by the method of HPLC. All the experimental mice were fasted for 24 h before experiment. PQ^{2+} (2.4% w/v PQ^{2+}; 100 μL/20 g of body weight) and C5AS·PQ^{2+} complex (12.1% w/v C5AS·PQ^{2+} complex; 100 μL/20 g of body weight) solutions were orally administered by eight mice, respectively; 0, 5, 15, 30, 45, 60, 90, and 120 min after the administration, blood was obtained by removing the eyeballs. This experiment was repeated three times to get the average value. Plasma was immediately prepared by centrifugation for 10 min at 4000 rpm (Sigma low-temperature centrifuge) for the following HPLC experiments. All experimental procedures were approved and in accordance with China's National Code of Animal Care for Scientific Experimentation.

HPLC analysis: HPLC experiments were performed on the Waters 600E HPLC system (Waters, USA) consisted of a manual injector, Waters 2487UV detector and Empower workstation. Plasma (200 μL) was mixed with 50 μL of 35% perchloric acid. The mixture was vortexed for 1 min (XW-80A Microvortex mixed Miriam) and centrifuged at 14000 rpm for 10 min (Sigma low-temperature centrifuge). Ten μL of the clear supernatant liquid was injected onto an HPLC column (KromasilC18, 250 mm × 4.6 mm, 5 μm) kept at room temperature. The mobile phase was a mixture of pH 2.37 phosphate aqueous solution/acetonitrile (75:25, V/V) containing 7.5 mM sodium 1-octanesulfonate, and the flow rate was 1 mL/min. The eluent was monitored with a UV−vis detector at 256 nm. The time-course of PQ^{2+} relative concentration in mice plasma after an oral administration of PQ^{2+} and C5AS·PQ^{2+} complex was gotten by calculating the peak area of the HPLC absorbance profile (at 256 nm) for all the mice plasma samples (Figure 9). HPLC chromatograms of PQ^{2+} in reference substance, blank plasma, and blood sample are shown (see Figure S5, Supporting Information).

Preparation of Crystal of C4AS (TC4AS) with DQ^{2+}. To an aqueous solution of C4AS (TC4AS) (0.05 mmol, 20 mL), 2 equiv of DQ^{2+} was added. Under stirring, 1 M HCl was dropped to adjust the pH to 1−2. Followed by filtration, the filtrate was placed to evaporation for 9 (13) days. Then the yellow crystal formed was collected along with its mother liquor for the X-ray crystallographic analysis.

The X-ray intensity data for complexes C4AS·DQ^{2+} and TC4AS·DQ^{2+} were collected on a Rigaku MM-007 rotating anode diffractometer, equipped with a Saturn CCD area detector system, using monochromated Mo Kα radiation at T = 113(2) K. Data collection and reduction were performed by program of Crystalclear. The structures were solved by using direct method and refined, employing full-matrix least-squares on F^2 (CrystalStructure, SHELXTL-97). X-ray structural data for C4AS·DQ^{2+}: $C_{52}H_{65}N_4O_{26.50}S_4$, M = 1298.32, triclinic, a = 13.699(5) Å, b = 13.796(5) Å, c = 15.189(6) Å, α = 88.402(11)°, β = 77.838(6)°, γ = 81.671(10)°, space group $P\bar{1}$, Z = 2, calculated density 1.553, crystal dimensions (mm^3): 0.18 × 0.16 × 0.16. μ = 0.267 mm^{-1}, $2\theta_{max}$ = 55.68°, 20355 measured reflections of which 12974 were unique ($R_{(int)}$ = 0.0725), final R indices [$I/\sigma(I) > 2$]: R_1 = 0.0710, wR_2 = 0.1784, R indices (all data): R_1 = 0.1057, wR_2 = 0.2027, GOF on F^2 1.001. X-ray structural data for TC4AS·DQ^{2+}: $C_{48}H_{44.50}N_4O_{20.25}S_8$, M = 1257.68, monoclinic, a = 12.6727(5) Å, b = 30.0947(13) Å, c = 13.5037(6) Å, α = 90°, β = 96.832(3)°, γ = 90°, space group $P1\,21/n\,1$, Z = 4, calculated density 1.634, crystal dimensions (mm^3): 0.36 × 0.22 × 0.16. μ = 0.436 mm^{-1}, $2\theta_{max}$ = 55.80°, 47613 measured reflections of which 12092 were unique ($R_{(int)}$ = 0.0503), final R indices [$I/\sigma(I) > 2$]: R_1 = 0.0474, wR_2 = 0.1119, R indices (all data): R_1 = 0.0561, wR_2 = 0.1175, GOF on F^2 1.058. CCDC-713042 and 713043 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223−336−033; or deposit@ccdc.cam.uk).

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Supporting Information Available: View showing the sacrificed mice and their pathological changes in lung and liver observed by the naked eye, HPLC chromatograms of PQ^{2+} in reference substance, blank plasma, and blood sample, crystallographic data in CIF format. This material is available free of charge via the Internet at http://pubs.acs.org.

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