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Elsinochrome A photosensitizers: Alternative drugs for photodynamic therapy

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Photodynamic therapy (PDT) has already been a multifunctional modality for various tumors and nontumorous diseases. However, the development of photosensitizers is relatively delayed, compared with the tremendous progress in laser technology. Elsinochrome A (EA), a perylenequinonoid pigment from China, has all the typical advantages of perylenequinones. Moreover, singlet oxygen quantum yield of EA is superior to other kinds of photosensitizers and EA could be artificially biosynthesized at present, which make it an alternative candidate for PDT. In this review, the photophysics, photochemistry, photobiology and chemical or biological syntheses of EA photosensitizers are briefly presented. Besides, the future prospects of EA photosensitizers are also proposed.

Keywords: Elsinochrome A; photodynamic therapy; photochemistry; photophysics; photobiology.

1. Introduction

Photodynamic therapy (PDT), distinguished from operation, chemotherapy or radiation therapy, is a comparatively nonintrusive therapeutic modality, which involves administration of a pathogenic tissue-localizing photosensitizer, followed by activation

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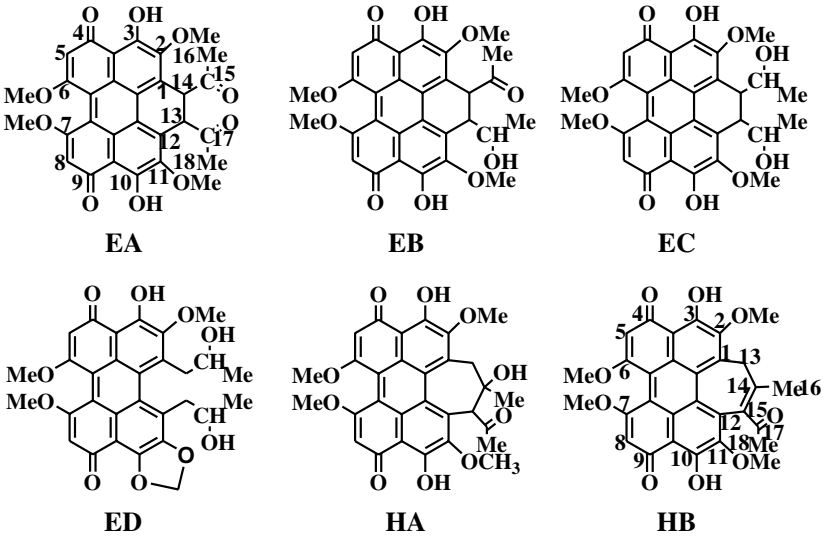

Fig. 1. The structures of EA, EB, EC, ED, HA and HB.

of the agent by laser and resulting in series of irreversible cytotoxic reaction in pathogenic tissue in the presence of oxygen.[1] PDT also has some advantages over other routine clinical treatments, such as more efficiency, more safety and lower toxicity for the surrounding normal tissues by more accumulation of photosensitizers and irradiation on the diseased targets.[2] Moreover, PDT can be administered repeatedly on the diseased targets for those diseases that will not become resistant to such treatment, and it can be combined with other clinical treatments for better clinical effects.[3] Nowadays, PDT has been an adjunctive therapy after routine treatments of several diseases, like melanoma.[4,5] And more efficiently, via combining PDT with radiotherapy, the required doses of photosensitizers, laser and radiation are greatly reduced, and significantly lessen the adverse side effects, which represents a potent avenue for treatment of diseases without specific remedies.[6–9]

There are three key elements that play a important role in PDT, including laser, oxygen and photosensitizers.[10] Despite rapid advances in laser technology, photosensitizers are still scarce nowadays. For clinical application, an ideal phototherapeutic agent should exhibit a uniform composition, high quantum yield of reactive oxygen species (ROS), low dark toxicity, selective localization in diseased tissues, short metabolic period, etc.[11] Compared with extensively studied porphyrin- and phthalocyanine-like photosensitizers, perylenequinonoid photosensitizers almost have the above virtues of perfect photosensitizers. For instance, hypericin, a well-known perylenequinone, has been considered to be a promising agent for PDT of superficial cancers or as a photoactive antiviral agent.[12,13]

Actually, many natural perylenequinonoid photosensitizers contain the structures of 4,9-dihydroxy-3,10-perylenequinones, like hypocrellins and elsinochromes from China. Hypocrellins including hypocrellin A (HA) and hypocrellin B (HB) (see Fig. 1), have been another worldwide studied perylenequinones.[14] Elsinochromes were firstly extracted from filamentous fungi, *Elsinoë*, including elsinochrome A (EA), elsinochrome B (EB), elsinochrome C (EC) and elsinochrome D (ED) (structures shown in Fig. 1).[15–17] Although hypocrellins and hypericin have been attracting intensive attention in the world, EA possesses the highest quantum yield of singlet oxygen among all the other perylenequinonoid photosensitizers.[18] Because the natural resources are limited, recently, it was reported that EA could be facially biosynthesized as the main product by solid state or submerged fermentation from laboratories.[19,20] which could effectively promote its potential application in PDT. So until now, more researches have been focused on this kind of photosensitizers. Herein, we attempt to make a brief review about the recent progresses of EA, mainly on the photophysics, photochemistry, photobiology and structural or biological modifications of EA.

2. Photophysics of EA

2.1. *The spectra property of EA*

The maximal absorption of EA in dimethyl sulphoxide (DMSO) solution is at 467 nm, and it also has two other absorption peaks at 528 and 570 nm, which are similar to those of hypocrellins, originated from the intramolecular proton transfer process (the absorption spectra are shown in Fig. 2). Meanwhile, the fluorescence emission spectra of EA, HA and HB are shown in Fig. 2. The maximum fluorescence of EA in DMSO is around 586 nm. The molar extinction coefficients of absorption spectrum and fluorescence quantum yield of EA are shown in Table 1, compared to those of HA and HB.[18]

It is believed that energy transfer from a triplet-excited photosensitizer to a ground-state oxygen molecule is a main pathway for ROS formation. So transient absorption spectra were measured for EA and HA in cyclohexane solution after a pulse excitation by the laser flash photolysis method.[18] Both of them have a large negative absorption ranging from 400 to 500 nm. The positive peaks of them appeared similarly, for EA at around 380, 510 or 610 nm, and 380, 520 nm or 610 nm for HA. Then the absorption decay of EA to time were derived, from which the triplet state lifetime of EA was estimated to be 5.2 μs, longer than that of HA or HB estimated to be 4.5 or 4.0 μs, respectively. This may be the reason why the single oxygen quantum yield of EA is larger than that of HA or HB. The T–T absorption spectrum of EA was also obtained with the absorption peak at around 390, 510 and 610 nm, respectively.

Table 1. The absorption spectra parameters and fluorescence quantum yield of EA, HA and HB in DMSO solution.

Compound	λ_{max}/nm (log ε)	Φ_F
EA	467(4.39), 528(4.03), 570(4.13)	0.11
HA	472(4.36), 543(4.05), 584(4.04)	0.11
HB	470(4.32), 549(4.01), 584(3.88)	0.08

2.2. *Partition coefficient and solubility of EA*

The partition coefficient (PC) value, determined by the ratio of a photosensitizer between n-octanol and PBS solution (pH = 7.4), is a quantitative measure for the amphiphilicity. The amphiphilicity of EA was experimentally evaluated by comparing its PC value and solubility in PBS solution (pH = 7.4) to those of HB.[21] The PC and solubility of EA in PBS are listed in Table 2, and those of HB are also included for comparison. It is shown that EA is even more hydrophobic than hypocrellins, which means

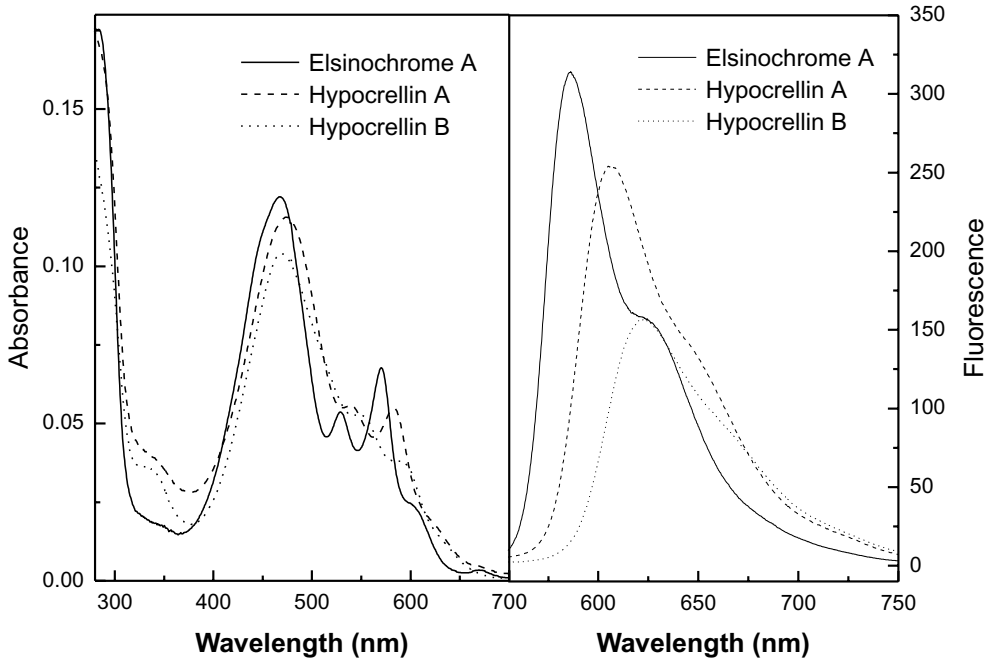

Fig. 2. Absorption spectra and fluorescence emission spectra of EA, HA and HB in DMSO solution.

Table 2. The PC and solubility in PBS.

Compound	PC	Solubility in PBS (μg/mL)
EA	34.5	1.4
HB	28.6	4.6

structural or biological modifications of EA are urgently needed.

2.3. *Theoretical calculation of the ground state of EA*

Taking advantage of the semiempirical method AM1, the structural, energetic and electronic properties of EA were evaluated, with those of HA as the reference.[22,23] From the results of geometry, the structure of EA is nearly symmetric and more planar than that of HA, which means the hexatomic ring of EA is rigid. But the difference in six-member ring of EA contributes little to the other results of theoretical calculation of EA. So the similar photosensitizing properties of EA and HA could be explained.

3. Photochemistry of EA

Similar to the photochemical properties of other perylenequinones, firstly, EA in ground state is excited to the singlet excited state under irradiation, and because there is a quick excited-state intramolecular proton transfer process in perylenequinonoid photosensitizers,[24–26] the singlet excited state of EA can be facilely converted to the triplet exited state by intersystem crossing. Then the triplet excited state of EA will undergo two types of reactions, known as Type I and Type II. In Type I process, an electron transfer occurs between the triplet exited state of EA and electron donor to form a semiquinone anion radical. Then superoxide anion radicals ($O_2^{\cdot-}$) will be produced by donation of an electron from the semiquinone anion radical to an oxygen molecule. The photogeneration of $O_2^{\cdot-}$ from EA has been proven in DMSO solution by theoretical and experimental researches.[21,23] As is known to all, once $O_2^{\cdot-}$ is formed, some other ROS, such as hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide, will be generated from perylenequinones by Fenton reaction.[27] However, since the water solubility of EA is too low, none of the direct electron paramagnetic resonance (EPR) signals of $^{\cdot}OH$ could be experimentally detected in aqueous solution.

While in Type II reaction, the triplet EA will straightly transfer its energy to a ground-state oxygen molecule, to form singlet oxygen ($^1O_2$). This process can be clarified through 9, 10-diphenylanthracene (DPA) (a typical $^1O_2$ receptor) photobleaching method.[18] No DPA bleaching was detected when EA, oxygen or laser was absent. When 1, 4-diazabicyclo[2,2,2]octane, a typical singlet oxygen ($^1O_2$) scavenger, was added to the reacting system, the photooxidation of DPA was seriously inhibited, which further confirmed that $^1O_2$ was formed in above experiments. By comparing the DPA bleaching rate of EA with that of HB, the $^1O_2$ quantum yield of EA is estimated to be 0.98, with HB as the reference.[18] And it is reported that by illuminating the $CHCl_3$ solution of EA and dibenzylamine for 6 min, a strong EPR signal of the oxynitride radical could be observed, generated by the reaction of dibenzylamine and $^1O_2$, which was formed by the photosensitization of EA.[28]

4. Photobiology of EA

4.1. *Photodynamic activities of EA on cells*

Photodynamic inhibitory efficiency of EA on human colorectal carcinoma Hce-8693 cells was evaluated.[29] Incubated with Hce-8693 cells at the concentration of 10^{-6} M, EA could lead to apoptosis of Hce-8693 cells. The apoptosis mechanism of Hce-8693 cells induced by photodynamic activity of EA was studied.[30] It was found that EA belonged to the cell cycle specific agent, and arrested Hce-8693 cell cycle in G1 phase to inhibit the proliferation of Hce-8693 cells. The ROS, generated after the photosensitization of EA, acted on and activated intracellular endonuclease, then induced the degeneration of DNA, cell apoptosis and inhibited the proliferation of carcinoma cells, to achieve the purpose of anti-tumor. The PDT of EA for animal entity tumor was also studied,[31] 90% illumination area of entity tumor became necrotic, with successive administration of EA for 14 days and time interval of 6 h before irradiation every day.

The PDT effect of EA on human umbilical vein endothelial cell line (ECV304) was investigated and compared with that of HB.[32] With the irradiation

condition of 532 nm laser, 20 J/cm^2, EA possessed a stronger photodynamic effect on ECV304 cells than HB. The IC_{50} (the photosensitizer concentration required to kill 50% of the cells) of EA or HB was 50.97 or 85.20 ng/mL, respectively. So, EA may be more promising for PDT treatment of microvascular diseased than hypocrellins.

4.2. *Antimicrobial activities of EA*

The EA mediated photodynamic antimicrobial efficacy on *Staphylococcus aureus*, *Bacilus subtilus*, *Escherichia coli* and *Proteus vulgaris* were examined *in vitro*.[33] The results showed that except for *E. coli*, EA exhibited strong photodamage to other tested bacterial strains, which signified that photodynamic activity of EA could effectively inhibit Gram positive bacteria.

4.3. *Photoinduced DNA cleavage activity of EA*

The interaction of EA and CT-DNA were studied by spectrophotometric method.[34] The results indicated that EA might be inserted into CT-DNA. When CT-DNA was added to EA, it suggested that the fluorescence quenching mechanism of EA by CT-DNA was static quenching, and when EA was added into CT-DNA and ethidium bromide (EB, a duplex DNA intercalator), the fluorescence intensity of CT-DNA-EB system greatly increased, which meant a strong interaction occurred between EA and CT-DNA-EB system. This is because the static binding of EA would change the space structure of the CT-DNA helix, and then EA gradually would replace EB from CT-DNA. Photoinduced damage of CT-DNA-EB by EA was also measured. 46.55% binding sites were damaged with irradiation on CT-DNA-EB system for 30 min. The main binding force between EA and CT-DNA was concluded to be electrostatic force, based on the calculations of the thermodynamics parameters ΔH and ΔS.

4.4. *The interactions between EA and biological proteins*

As a potential phototherapeutic agent, the interactions of EA and the main biological proteins *in vivo* should be studied, which could provide some useful information for interaction of drugs to biological targets and rational drug administration. The interaction of EA with hemoglobin or myoglobin has been investigated under the irradiation or dark condition through spectroscopic methods.[35,36] It was concluded that the conformation of hemoglobin or myoglobin was changed due to the interaction of EA with hemoglobin or myoglobin. But the binding sites were variant under the above conditions. Without the irradiation, EA only bound to the surface amino acid of hemoglobin or myoglobin. But with the illumination, EA interacted with both amino acid and the interior hemachrome of hemoglobin or myoglobin, and the binding force between EA and hemoglobin or myoglobin was mainly hydrophobic interaction. Fluorescent spectra of hemoglobin or myoglobin were quenched by EA, and the quenching mechanism was determined to be static quenching.

4.5. *The pharmacokinetics of EA in vivo*

The tissue distribution and excretion kinetics of EA in mouse model have been studied. After drenching rats with EA (30 mg), the concentrations of EA contained in liver, kidney, lung, brain, feces and urine were determined through high-pressure liquid chromatography (HPLC) method.[37] It turned out that the distribution concentration in different tissues decreased in order: liver>lung> kidney>brain. None of EA could be detected in urine or brain. After 24 h, the cumulative excretion of EA in feces was $(83.69 \pm 3.91)\%$. Therefore, the main distribution tissues of EA *in vivo* were liver, lung and kidney; and EA was excreted mostly in feces by live and intestinal pathways.

4.6. *The acute toxicity of EA*

The acute toxicity of EA was also examined as a reference for safe administration. Its acute toxicity was recorded for two weeks after normal mice had taken high concentration of EA.[38] After observing for two weeks, all the experimental animals were alive, and no obvious side effects were shown. The maximum tolerated dose of EA was 5 mg/Kg, equivalent to 253 times of adult daily dosage. Hence, the toxicity of EA is small, and EA can be safely used for clinic.

5. Biological or Structural Modifications of EA

Previously, it was reported that EA could cause tautomerization, oxido-reduction complexation reaction, methylation, diacetylation and four acetylation of EA,[39] so the chemical properties of EA is relatively reactive. Because EA is nearly hydrophobic, the water solubility that improved modifications of EA have always been a cardinal task. Meanwhile, for PDT of cancers, the tiny red absorption of this photosensitizer should also be ameliorated. Owing to similar chemical features, extensive experiences of biological or chemical modifications of hypocrellins or hypericin should be introduced into the modifications of EA. Herein, the biological or chemical modifications of EA were briefly described as follows.

5.1. *Biological modifications of EA*

Preparation of biocompatible nanoparticles was thought to be one of the effective strategies for lipophilic drug delivery *in vivo*. However, until now, there are not many studies about the biological modifications of EA. A water-soluble silica EA nanosphere was prepared by sol–gel method.[40] The nanosphere was much more soluble than free EA. And the singlet oxygen yield of this nanosphere was much more than that of free EA in water. Meanwhile, the nanosphere exhibited the features of controlled release of attached EA, which suggested that this formulation may be potential for PDT of tumors. Then a water-soluble nanocolloid embedded with EA was prepared by using single 3-aminopropyltriethoxysilane.[41] The photostability and solubility of this formulation were greatly improved compared to free EA. This formulation could efficiently generate singlet oxygen, and the photocleavage ability of this formulation to DNA was even more than that of free EA. However, the bioevaluation of the above formulations should be further researched. Actually, appropriate drug-delivery vehicles of EA will greatly impulse the clinical application of EA, so more efforts should be involved in the preparation of water-soluble EA nanoparticles.

5.2. *Chemical modifications of EA*

Alternatively, in order to obtain strong absorption in phototherapeutic window (600–900 nm) and water-soluble compounds, the other practical strategy is structural modification of EA. However, the lipophilicity of EA is the key for keeping their cellular affinity and PDT activity, but hydrophilicity of drugs is the necessary prerequisite for clinical administration. But how to find a novel derivative to achieve the balance between lipophilicity and hydrophilicity, i.e., amphiphilicity, has always been a hot potato. Previously, brominated EA derivatives (**1**) were obtained at room temperature.[42] The reactions were shown in Fig. 3. Apparently, the derivatives are too lipophilic for direct usage.

Actually, amphiphilicity should be quantitative, but not qualitative, i.e., a drug could be directly administered for intravenous injection, while the lipophilicity of the drug is kept as high as possible. Based on these, 3-mercapto-1-propanesulfonic acid was introduced into the 5th site of EA to gain compound **2**, shown in Fig. 4.[21] Thanks to the almost symmetric property of EA, structural modifications at 5 and 8 positions obtained the identical derivatives, but those of hypocrellins were two kinds of derivatives. Therefore, the synthesis of these kinds of EA derivatives were extremely simplified and the yield of **2** was 40%. The solubility and PC of **2** were estimated to be 5.1 mg/mL and 7, respectively, which indicated that **2** could directly be used for intravenous injection, and maintain high affinity to biological targets. The singlet oxygen quantum of **2** was 0.73, close to that of HB. *In vitro* studies showed that the photodynamic activity of **2**

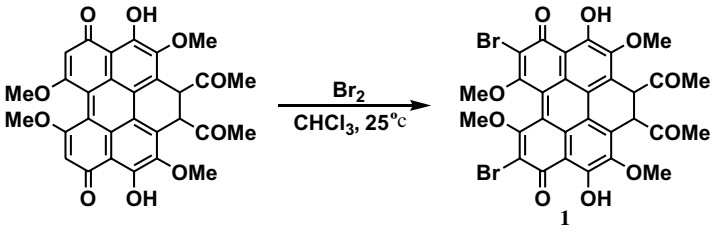

Fig. 3. Synthesis of **1**.

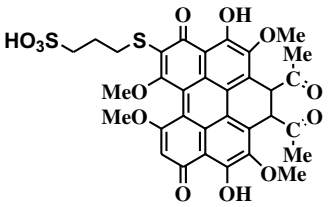

Fig. 4. The structure of **2**.

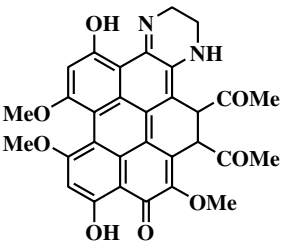

Fig. 5. The structure of **3**.

was as high as 60% of EA, which was higher than most hypocrellin derivatives. So, **2** was expected to be a hopeful drug for PDT. Recently, ethylenediamine-modified EA (**3**, Fig. 5) was designed and synthesized.[43] The absorption of **3** red-shifted greatly, and the main absorption peak of **3** was at 708 nm. It could effectively generate superoxide anions and singlet oxygen, and the photodamage of **3** to DNA was apparently stronger than that of EA. Nevertheless, the solubility of **3** was not sufficient to enable directly the dissolution for intravenous injection.

6. Future Trends

Currently, only some rudimental researches have been done for EA, and the applications of EA may be lagged due to the lack of natural ingredients of EA and easy photobleaching under visible and ultraviolet lights.[44] However, EA could now be facilely biosynthesized with a yield of 0.2%[18] and the ability of generation $^1O_2$ of EA is almost the highest compared to other kinds of photosensitizers. As is well known, the high efficiency of PDT usually refers to singlet oxygen generation mechanism. Therefore, applied foreground of EA will be enormous. However, the almost insoluble feature of EA becomes the stumbling block for its clinical application.

Nowadays, a powerful and effective pharmaceutical strategy is preparation of water-soluble EA nanoparticles. For instance, we could make attempts to prepare EA liposomes in the future. And as everyone knows, the only one photosensitizer clinically approved for PDT treatment of AMD — Visudyne® is the liposome formulation. Liposomes are artificial membranes usually composed of biocompatible phospholipids. So the side effects of these formulations were usually negligible, and they could transport embedded drugs into the target tissues. It was reported that the hypocrellin liposomes were tested to accumulate more tumor tissues or microvasculatures than free hypocrellins *in vivo*,[45,46] and to keep most PDT effects of their parents. Besides, the PDT efficacy of hypericin liposomes was also enhanced in contrast to the free hypericin *in vitro* and *in vivo*.[47] Since the natural photosensitizing activity of EA is even higher than that of hypocrellins or hypericin, preparation of EA liposomes will represent a new emerging field for PDT.

On the other hand, another chemical strategy is to design and synthesize EA photosensitizers, and the aim of chemical medication is to tune the amphiphilicity of EA derivatives. According to the former researches on hypocrellins or hypericin, it seems to conclude that the highly water-soluble modified perylenequinonoid derivatives could hardly retain their PDT activities for these natural lipophilic perylenequinones.[48,49] Besides, lipophilic photosensitizers are in high tumor tissue affinity, which ensure their high photodynamic activities. So finding optimized amphiphilicity, was vitally important for structural modifications of EA, which is one effective method to achieve its application for PDT. Not long ago, a series of amino alkyl sulfonic acid substituted hypocrellin derivatives were synthesized, evaluated,[50–52] and 17-5-amino-1-pentanesulfonic acid substituted HB derivative (**4**, Fig. 6) was sought out as achieving optimized amphiphilicity. We may also find some optimized amphiphilic EA derivatives for PDT, by borrowing these methods of gradually adjusting the length of the carbon chain in substituents. Unlike hypocrellins, different sited substituted hypocrellin derivatives differ greatly in generation abilities of singlet oxygen.[53] Because of the naturally high photosensitizing activity of EA, whether substituents introduce into the perylenequinonoid ring or the six-member ring of EA, the resulted EA photosensitizers may possess high PDT efficacy. Moreover, design and synthesis of new EA photosensitizers should be

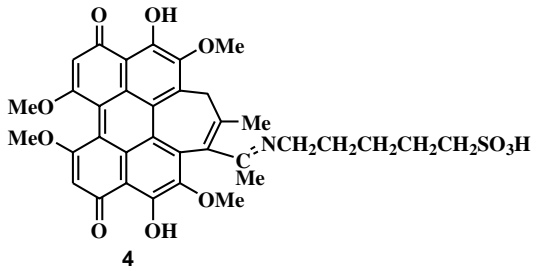

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Fig. 6. The structure of **4**.

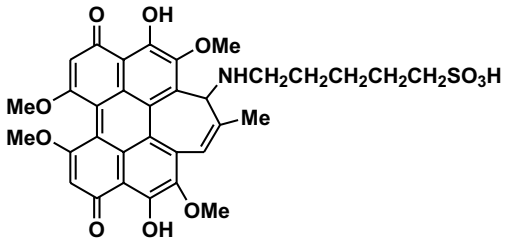

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Fig. 7. The structure of **5**.

based on the target characters of diseases, because applying medicines against the diseases could improve their curative effects. Taking hypocrellin derivatives as examples, water-soluble amino-alkylsulfonic acid substituted hypocrellin derivative specially designed for PDT of AMD (**5**, Fig. 7), has been synthesized and authenticated to bring about more damage to the blood vessels than its parent *in vivo*.[54] Thus, these above experiences of synthesized hypocrellin derivatives could help us to avoid detours toward the structural modifications of EA.

7. Conclusion

In this paper, the research status and future prospect of EA photosensitizers were briefly reviewed. The strongest generation ability of singlet oxygen, high phototoxicity against tumor cells, vascular endothelial cells or bacteria, low toxicity in dark and fast metabolic rate *in vivo*, make EA photosensitizers promising drugs for PDT. However, like other perylenequinones, EA is hydrophobic, which may be solved by preparing the pharmaceutical formulations or structural modifications of EA, according to the target characters of different diseases. Furthermore, the design and evaluation of new EA photosensitizers should be the team works from chemists, biologists and medics, and we hope more invested funds will be injected into these fundamental researches.

Acknowledgments

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