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Syntheses of 7-dehydrocholesterol peroxides and their improved anticancer activity and selectivity over ergosterol peroxide†

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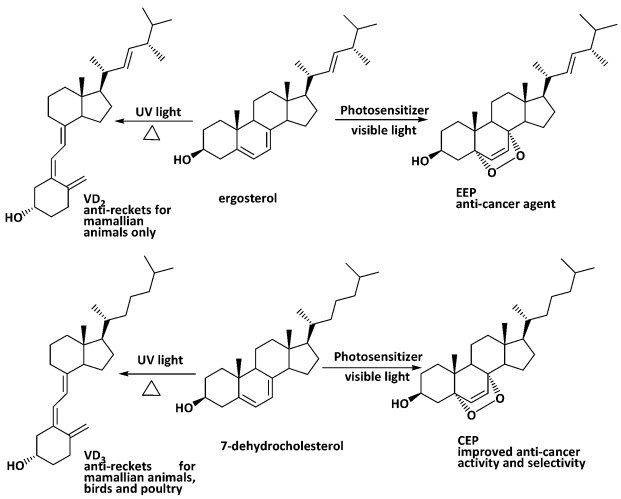
7-Dehydrocholesterol peroxide (5α,8α-epidioxycholest-6-ene-3β-ol, CEP) and its acetate and hemisuccinate derivatives were synthesized and isolated for the first time, which exhibit improved anticancer activity and selectivity over ergosterol peroxide (5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol, EEP), showing potential as new chemotherapeutic agents.

Endoperoxides are promising pharmacophores for antimalarial, anticancer, and antiviral agents.[1] One of the most prominent examples is artemisinin, which is a sesquiterpene lactone endoperoxide and exhibits excellent antimalarial activity through homolytic cleavage of the O–O bond induced by endogenous reductants.[2] Similar to artemisinin, ergosterol peroxide (5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol, EEP) has also shown a variety of biological effects, such as anti-inflammatory,[3] antibacterial,[4] and antitumor activities,[5] and as a result has received much attention as a new drug candidate. Although EEP is widely found in mushrooms,[6] plants,[7] and lichens,[8] the most facile and economic way to obtain it in large quantity is photosensitized oxidation of ergosterol (Scheme 1).[5c,9]

As an analogue of ergosterol, 7-dehydrocholesterol (7-DHC) plays important roles in animals. It is the bio-synthetic precursor of vitamin D_3 and is present in relatively high concentrations in skin, in which it is converted to vitamin D_3 upon UV irradiation (Scheme 1).[10] Ergosterol may also undergo similar photochemical conversion to vitamin D_2 upon exposure to UV light. Interestingly, though vitamin D_3 and vitamin D_2 are very similar in the treatment of rickets for people and mammalian animals, the therapeutic effect of vitamin D_3 is much higher than that of vitamin D_2 for poultry and birds. As a result,



Scheme 1 Photochemical conversions of ergosterol and 7-DHC and biological effects of the products.

one may expect that 7-dehydrocholesterol peroxide (5α,8α-epidioxycholest-6-ene-3β-ol, CEP) may present different biological effects with respect to its analogue of EEP. It is really a surprise that there are very few studies on the photosensitized oxidation of 7-dehydrocholesterol compared to a large quantity of research on the synthesis and isolation of EEP and its biological effects. P. W. Albro and L.-Z. Wu's groups reported the synthesis of CEP from 7-DHC, however, a 3 : 1 mixture of CEP and CHP (5α-7-hydroperoxy-5,8-diene-3β-ol) was obtained and no further isolation of CEP was performed.[11] Up to now, there is no report associated with the biological effects of CEP. Such a gap inspired us to explore the photochemical synthesis and biological effect of CEP. Moreover, the 3β-hydroxyl group of sterols provides a versatile handle for chemical modification to finely tune their biological properties, for example, glucose conjugation of EEP led to an improved proliferation inhibition over many tumor cell lines,[12] and cholesterol hemisuccinate was found to alter the function of P-glycoprotein (Pgp), a protein that has been linked to the emergence of multidrug resistant

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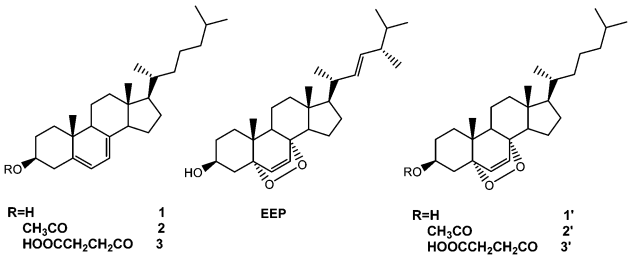

Scheme 2 Structures of **1–3**, **1′–3′**, and EEP.

(MDR) cancer.[13] Thus, CEP acetate and CEP hemisuccinate (**2′** and **3′** in Scheme 2) were also photochemically synthesized and their anticancer activity was compared with that of CEP and EEP. Encouragingly, CEP and its hemisuccinate derivative display much improved *in vitro* anticancer activity against human breast cancer cells SKOV-3, cervical carcinoma cells HeLa, lung cancer cells A549 and prostatic carcinoma cells DU145 and diminished cytotoxicity toward human normal liver cells L-02 with respect to EEP.

The photooxidation reaction of 7-DHC was carried out at 0 °C in different O_2-saturated solvents using *meso*-tetraphenylporphyrin (TPP) as the photosensitizer and a high-pressure Hg light equipped with a 400 nm cut-off filter as the light source. As shown in Table 1, after full consumption of 7-DHC, the isolated yields of CEP followed an order of pyridine > *n*-hexane > benzene > CH_2Cl_2 (entries 1–4). Notably, when a mixed solvent of *n*-hexane and methanol was used as the medium, improved yields were obtained. At the volume ratio of 3 : 1 for *n*-hexane/methanol, the isolated yield of CEP was as high as 81% (entries 5–8). While increasing TPP did not give rise to further improvement for the photoreaction, the reduced TPP from 0.1 to 0.05 equiv. led to a lower yield (entry 9). Other single oxygen photosensitizers, such as hematoporphyrin, eosin Y, and methylene blue, can also realize the transformation in *n*-hexane/methanol (3 : 1), but with lower yields than the case of TPP (entries 10–12). Their poor performance may mainly due to their bleaching during the photoreaction. In sharp contrast, the recycled TPP from the entry 6 can be reused to give a yield of 73% (entry 13). As expected, elevated reaction temperature is unfavourable for the production of CEP (entry 14).

Table 1 Reaction optimization for the synthesis of CEP[a]

Entry	Photosensitizer (mol%)	Solvent	T (°C)	t^b (h)	Yield[c] (%)
1	TPP (0.1)	Pyridine	0	2	63
2	TPP (0.1)	Benzene	0	2	58
3	TPP (0.1)	CH_2Cl_2	0	1	43
4	TPP (0.1)	*n*-Hexane	0	3	62
5	TPP (0.1)	Mix (4 : 1)[d]	0	3	69
6	TPP (0.1)	Mix (3 : 1)[d]	0	3	81
7	TPP (0.1)	Mix (2 : 1)[d]	0	3	74
8	TPP (0.1)	Mix (1 : 1)[d]	0	3	70
9	TPP (0.05)	Mix (3 : 1)[d]	0	3	64
10	Hematoporhyrin (0.1)	Mix (3 : 1)[d]	0	3	56
11	Eosin Y (0.1)	Mix (3 : 1)[d]	0	3	40
12	Methylene blue (0.1)	Mix (3 : 1)[d]	0	3	62
13	TPP (0.1)[e]	Mix (3 : 1)[d]	0	3	73
14	TPP (0.1)	Mix (3 : 1)[d]	30	3	51

[a] Reaction conditions: 1.56 mmol 7-DHC and 1.9 μM photocatalyst in 20 mL of solvent was subjected to visible light irradiation (≥400 nm) under magnetic stirring and bubbling with oxygen. [b] By which all substrate was consumed. [c] Isolated yield. [d] Mixed solvent of *n*-hexane/methanol with varied volume ratio. [e] Recycled TPP from entry 6 was reused.

A similar trend was also found in the preparation of EEP from ergosterol (Table S1, ESI†). The use of *n*-hexane/methanol (3 : 1) as solvent led to an enhanced yield of EEP with respect to the literature results.[5c,11a] Besides a higher isolated yield of CEP or EEP, the mixed solvent of *n*-hexane/methanol we used is more economic and environmentally friendly than the solvents, such as pyridine, benzene, and CH_2Cl_2, that have been widely used in the photochemical syntheses of EEP.[5c,11a] 7-Dehydrocholesterol acetate and 7-dehydrocholesterol hemisuccinate (**2** and **3** in Scheme 2) were also synthesized and their corresponding peroxides **2′** and **3′** were prepared photochemically in the yields of 78% and 81%, respectively (Table S1, ESI†).

The chemical structure of CEP (**1′**) and its acetate and hemisuccinate derivatives (**2′** and **3′**) were fully characterized by high-resolution ESI-MS, ^{1}H NMR, ^{13}C NMR, HMBC, and COSY through comparison with EEP and other structurally related sterol endoperoxides (Fig. S1–S30, ESI†).[14] Taking CEP as an example, the ESI-MS ion peak of CEP at *m*/*z* 439.3161 is in good agreement with $[M + Na]^+$. The assignments of all ^{13}C signals and partial ^{1}H signals of CEP and EEP are compiled in Tables S2 and S3 (ESI†), and those of EEP are in good agreement with the reported values.[14c] These chemical shift assignments get full support from the HBMC spectra. For example, one olefin proton at 6.24 ppm (H7, see atom numbering in Fig. 1) of CEP showed correlations with C5, C6, C8, C9 and C14 in its HMBC spectrum, while the other olefin proton at 6.51 ppm (H6) correlated with C4, C5, C7, and C8. The chemical shifts of both olefin protons and their coupling constant of 8.4 Hz, in combination with two oxygenated quaternary carbons at C-5 (82.3 ppm) and C-8 (79.5 ppm), support the 5α,8α-epidioxy structure in the B ring.[14a] Furthermore, the HMBC spectrum may aid in the differentiation of H-18 (singlet) from H-19 (singlet) by its coupling to C17, an atom that is also coupled to H-21 (doublet).

MTT assay was used to evaluate the cytotoxicity of EEP, CEP (**1′**) and its two derivatives (**2′** and **3′**) toward human lung cancer cells A549, breast cancer cells SKOV-3, cervical carcinoma cells HeLa and prostatic carcinoma cells DU145, as well as human normal liver cells L-02. 7-DHC (**1**) and its two derivatives (**2** and **3**) were also tested as negative controls. Fig. 2 shows the

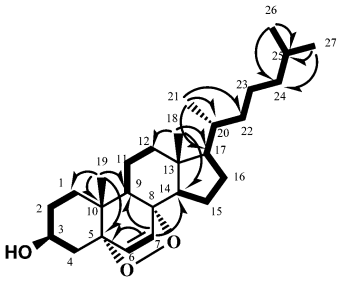

Fig. 1 Atom numbering and key COSY and HMBC correlations of CEP.

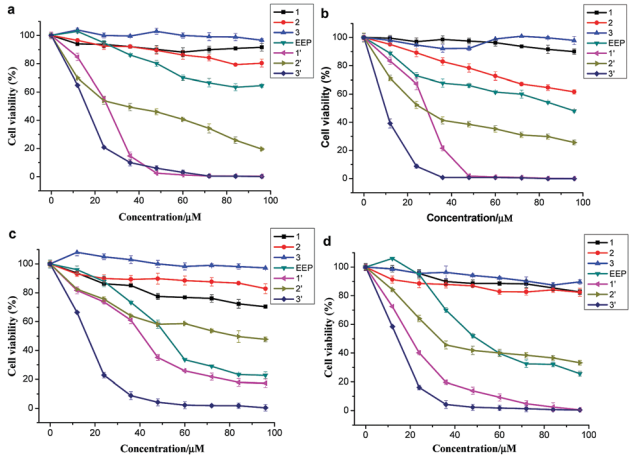

Fig. 2 Cytotoxicity of **1–3**, **1′–3′** and EEP against A549 (a), SKOV-3 (b), HeLa (c), and DU145 (d) cells. Data are presented as means ± S.D. of triplicate samples.

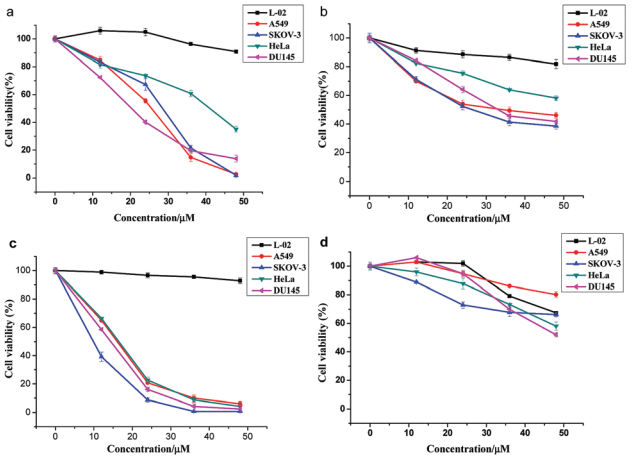

Fig. 3 Cytotoxicity selectivity of **1′** (a), **2′** (b), **3′** (c) and EEP (d) against various tumor cells over normal L-02 cells. Data are presented as means ± S.D. of triplicate samples.

cell viability of the examined tumor cells at 37 °C after 48 h of incubation with varied concentrations of the examined compounds, and the IC_{50} values are collected in Table 2. While both CEP and EEP showed cytotoxicity against the examined tumor cells, the *in vitro* anti-cancer activity of CEP is significantly larger than that of EEP. Taking A549 cells as an example, they may be totally inactivated at around 40 μM of CEP, however, the cell viability was still as high as 80% when incubated with EEP at the same concentration. Such results reveal the important role of the side chain of the sterol endoperoxides on their biological effects. Additionally, the lack of cytotoxicity of 7-DHC implies that the anticancer activity of both CEP and EEP may result from their endoperoxide moieties, which was further confirmed by comparing the cytotoxicity of **2′** and **3′** with respect to that of **2** and **3**. Interestingly, though the acetate derivative of CEP (**2′**) generally showed lower anticancer activity than CEP, its hemisuccinate derivative indeed exhibited an improved inactivation ability over the all examined tumor cell lines, demonstrating the importance of the chemical modification by way of the 3β-OH group. Notably, both CEP and its hemisuccinate derivative display improved cancer selectivity than EEP, as evidenced by much diminished cytotoxicity toward L-02 cells as shown in Fig. 3, suggesting their promising potential as new chemotherapeutic agents.

Table 2 IC_{50} values of **1–3**, **1′–3′** and EEP

	IC_{50} (μM)			
Compound	A549	SKOV-3	HeLa	DU145
1	>100	>100	>100	>100
2	>100	>100	>100	>100
3	>100	>100	>100	>100
1′	25.6 ± 0.1	28.5 ± 0.1	41.1 ± 0.2	20.4 ± 0.2
2′	34.4 ± 0.1	26.6 ± 0.1	83.0 ± 0.2	33.2 ± 0.2
3′	16.1 ± 0.02	9.9 ± 0.02	16.5 ± 0.02	14.4 ± 0.02
EEP	>100	92.3 ± 0.8	51.9 ± 0.2	50.0 ± 0.5

A549, SKOV-3, HeLa and DU145 cells were treated with varied concentrations of the examined compounds for 48 h. Data are presented as means ± S.D. of triplicate samples.

In summary, CEP and its two derivatives were synthesized and isolated in high yields for the first time by the photosensitized oxidation approach in a mixed solvent of *n*-hexane/methanol (3 : 1, v/v). Compared to EEP, CEP and its hemisuccinate derivative exhibit much improved anticancer activity and selectivity, showing promising application potential in cancer chemotherapy.

Experimental

Syntheses of CEP and its derivatives. A solution of **1**, **2**, or **3** (1.56 mmol) and TPP (1.9 μmol) in 20 mL hexane/methanol (3 : 1) was irradiated for 3 h at 0 °C under magnetic stirring and continuous bubbling with oxygen. A 500 W high-pressure Hg lamp in combination with a 400 nm cut-off glass filter was employed as the light source. The crude product was purified on silica gel using *n*-hexane/ethyl acetate (3 : 1 in volume ratio) as eluent.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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