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Synthesis and biological evaluation of novel steroidal 5α,8α-endoperoxide derivatives with aliphatic side-chain as potential anticancer agents

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ABSTRACT

By inspiration of significant anti-cancer activity of our previously screened natural ergosterol peroxide (EP), a series of novel steroidal 5α,8α-endoperoxide derivatives **5a–d** and **14a–f** were designed, synthesized, and biologically evaluated for their *in vitro* anti-proliferative inhibitory and cytotoxic activity. The results revealed that most of these compounds showed moderate-to-excellent anti-proliferative effects against the tested cancer cell lines (*i.e.* HepG2, SK-Hep1, MDA-MB-231 and MCF-7). Among them, compound **5b** and **14d** exhibited preferable inhibitory activities (IC_{50} of **5b** and **14d** are 8.07 and 9.50 μM against HepG2, respectively). The structure-activity relationships indicated that incorporation the peroxidic bridge to the steroid scaffolds at C-5 and C-8 positions together with the aliphatic side-chain at the C-17 position would provide synergistic effect for the bioactivity.

1. Introduction

Nowadays, natural drugs have attracted extensive attention in health promotion and disease treatment including cancer [1,2]. In addition, natural product-based drug discovery is a major route leading to developing therapeutic drugs for various diseases. Many natural health products are obtained from plants, animals, and microorganisms. Natural endoperoxides are cyclic organic compounds, with an O-O single bond as a peroxidic bridge [3,4]. They play an important role in drug synthesis as well as in medicine, and represent the central part of artemisinins, outstanding antimalarial drugs, honored with the Noble Price in Medicine 2015 [5]. Although best known as potent antimalarials, cyclic peroxides also exhibit a range of activities which encompasses antifungal, antiviral and anticancer activity [6,7]. Among natural endoperoxides, steroidal 5α,8α-endoperoxides are the important active lead compounds in drug discovery, which are well known for their 5α,8α-peroxy moiety [8]. Ergosterol peroxide (5α,8α-epidioxyergosta-6,22-dien-3β-ol, EP) (Fig. 1), is a member of a class of fungal secondary metabolites of sterol 5α,8α-endoperoxide derivatives [9]. It can be isolated from many medicinal fungi, such as *Sarcodon aspratus*, *Hericium erinaceum*, *Armillariella mellea*, *lactarius hatsudake*, *hypsizigus marmoreus*, et al. [10–12]. It has been reported that EP can inhibit tumor growth by anti-angiogenesis or cytotoxicity [13–15].

In our previous study, we found that EP purified from *Ganoderma lucidum*, induced cell death and inhibited cell migration, cell cycle progression, and colony growth of human hepatocellular carcinoma cells [16]. We further examined the mechanism associated with this effect and found that treatment with EP increased expression of Foxo3a mRNA and protein in HepG2 cells. The levels of Puma and Bax, pro-apoptotic proteins, were effectively enhanced. Our results suggest that ergosterol peroxide stimulated Foxo3 activity by inhibiting pAKT and c-Myc and activating pro-apoptotic protein Puma and Bax to induce cancer cell death. With further clinical development, EP represents a promising new reagent that can overcome the drug-resistance of tumor cells [17].

As an important active lead compound in drug discovery, EP is well known for its 5α,8α-peroxy moiety. We have recently developed a simple and practical synthetic route to obtain novel series of sterol 5α,8α-endoperoxides from readily available natural sterols. The synthesis of endoperoxides achieved by the reaction of steroidal $\triangle^{5,7}$-diene intermediates with singlet oxygen ($^1O_2$), which can be conveniently generated photochemically using eosin Y (EY) as a

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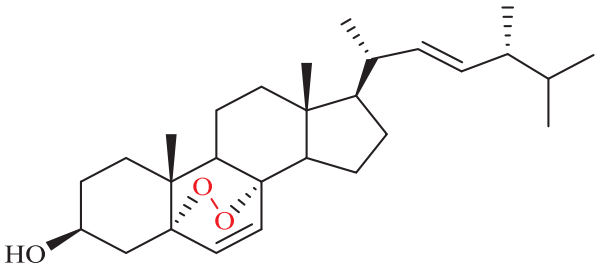


Fig. 1. Structure of ergosterol peroxide (EP).

photosensitive catalytic oxidizer from molecular oxygen (photooxygenation) [18–20]. Acknowledging the limited structure-activity relationship studies for EP, we preliminary designed and synthesized a series of novel steroidal 5α,8α-endoperoxide derivatives that with C-17 aliphatic side chain. Meanwhile, their biological activities (IC_{50} values) were compared using a MTT assay with four human cancer cell lines. We hope to get valuable information for further design of novel steroidal anticancer agents. Herein, we designed and synthesized a series of novel steroidal 5α,8α-endoperoxide derivatives that with C-17 aliphatic side chain.

2. Result and discussion

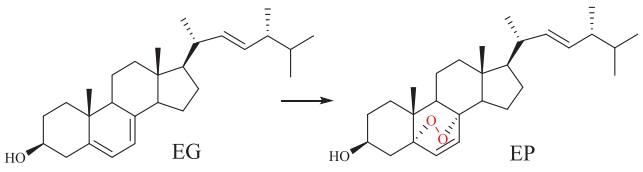
2.1. Chemistry

2.1.1. Synthesis of EP from ergosterol

Using natural ergosterol (EG) as the starting material, we performed chemical synthesis and purification as described in Scheme 1. EP was purified as white crystalline needles. The photooxygenation reaction is the key step of the whole synthetic route and its reaction conditions had to be optimized in order to get high conversion. A typical experimental procedure for the optimization of the photooxygenation reaction conditions was described using EG as an example (Table 1). On the basis of the optimized reaction conditions, we synthesized EP from EG with eosin Y in pyridine, irradiated with iodine tungsten lamp and kept bubbling oxygen for 0.5 h to get EP. Eosin Y (EY) was selected as the photosensitizer, and other conditions such as the solvent, reaction time, reaction temperature and light were chosen based on the results shown in Table 1.

Crystal of EP suitable for single-crystal X-ray diffraction was firstly obtained by slow crystallization from *n*-hexane/ethyl acetate solution at ambient temperature (Fig. 2). An interesting feature in its structure is the presence of two molecules in the crystallographic asymmetric unit. The structure confirms the α-stereochemistry of the peroxy bond at C-5 and C-8 positions. Crystal data and structure refinement details for EP was presented in Table S1. (CCDC numbers 1502457).

A plausible reaction mechanism for the formation of 5α,8α-peroxy moiety from steroidal $\triangle^{5,7}$-diene intermediate is depicted in Fig. 3. First, singlet oxygen is generated from sensitization by eosin Y^* (EY^*). Then, the clear region-selectivity of the singlet oxygen attacks to the C-5 and C-8 positions of the conjugated double bond system in the [4 + 2] cycloaddition manner. What's more, the α-π-facial stereo-selectivity of



Scheme 1. Synthesis of EP from ergosterol. Reagents and conditions: O_2, eosin Y, pyridine, *hv*, 0 °C, 0.5 h.

Table 1
Optimization of the photooxygenation reaction conditions.

Entry	Reaction system[a]	Temperature[b]	Light[c]	Oxygen source[d]	Time (h)	Yield (%)[e]
1	EG-EY-Py	Ice-water bath	100 W	O_2	0.5	22
2	EG-EY-Py	Ice-water bath	300 W	O_2	0.5	46
3	EG-EY-Py	Ice-water bath	500 W	O_2	0.5	62
4	EG-EY-Py	Ice-water bath	500 W	O_2	1.0	67
5	EG-EY-EtOH	Ice-water bath	500 W	O_2	0.5	54
6	EG-EY-Py	Ice-water bath	500 W	Air	0.5	—
7	EG-EY-Py	RT	Daylight	O_2	0.5	—
8	EG-EY-EtOH	RT	Daylight	Air	5	7
9	EG-EY-EtOH	RT	Daylight	Air	10	18

[a] EG: ergosterol; EY: eosin Y; Py: pyridine; EtOH: anhydrous ethanol.
[b] RT: Room temperature.
[c] Light: Iodine-tungsten lamp 220 V (100, 300, 500 W).
[d] O_2: high purity oxygen (> 99.995%).
[e] Isolation yield.

the reaction of singlet oxygen with $\triangle^{5,7}$-diene intermediates is well accepted owing to the fact that the methyl group at C-10 is β (axial) (Fig. 3, 19-CH_3).

2.1.2. Synthesis of *5a–d* from natural steroids

Using natural β-sitosterol (**1a**), cholesterol (**1b**), pregnenolone (PREG, **1c**), and dehydroepiandrosterone (DHEA, **1d**) as the starting materials, we performed chemical synthesis and purification as described in Scheme 2. Four new 5α,8α-endoperoxides **5a–d** were synthesized. Compounds **2a–d** were prepared *via* acetylation reaction of **1a–d**, which then underwent bromination and debromination with NBS, *n*-Bu_4NBr and *n*-Bu_4NF to afford $\triangle^{5,7}$-diene acetates **3a–d**. Subsequently, products **4a–d** were obtained after deacetylation reaction of **3a–d**. Finally, the target steroidal 5α,8α-endoperoxide derivatives **5a–d** were obtained by optimized photooxygenation method in Table 1 (entry 3).

2.1.3. Synthesis of *14a–f* from DHEA

Using readily available DHEA (**1d**) as the starting material, we performed chemical synthesis and purification ergosterol peroxide analogues **14a–f** as described in Scheme 3. The synthesis started with 3β-(*tert*-butyldimethylsilyl)oxy-androst-5-ene (**6**), which is readily available from **1d**. The side chain with natural configuration at C-17 and C-20 was introduced by the method developed by Uskokovic et al. [21]. The Wittig reaction of **6** with ethyltriphenylphosphonium bromide stereoselectively gave the *Z*-olefin **7** with a trace of the isomeric olefin in quantitative yield. Due to the difficulty in separating the stereoisomers, the mixture of the isomers was used in the next step. The ene reaction of the thus-obtained olefins with paraformaldehyde in the presence of a catalytic amount of boron trifluoride etherate afforded the alcohol **8** stereo-specifically in 86% yield. The epimer at C-20, derived from the *E*-olefin, was cleanly separated by column chromatography. Catalytic hydrogenation of **8** over 5% Pt-C reduced only the $\triangle^{16}$-double bond stereoselectively to give the mono-olefin **9** in quantitative yield. Formation of the $\triangle^{5,7}$-diene moiety was best achieved by Rappoldt et al. [22], successive treatment of **9** with NBS, *n*-Bu_4NBr and *n*-Bu_4NF produced the diene **10** in 26% yield. The diene moiety is stable enough to allow further elaboration, as described in Scheme 3. Tosylate **11** was readily prepared from alcohol **10** in high yield. Then, alkylation of the intermediate **11** with different Grignard reagents to get aliphatic side chain analogues **12a–f** [23]. The analogues **12a–f** were further treated with *n*-Bu_4NF to produce the intermediates **13a–f**. Finally, the target steroidal 5α,8α-endoperoxide analogues **14a–f** were obtained by optimized method in Table 1.