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ARTICLE

Synthesis and biological activity evaluation of novel peroxo-bridged derivatives as potential anti-hepatitis B virus agents

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Previous studies have demonstrated that natural steroid compounds containing peroxide bridge exhibited potential anti-hepatitis B virus activity. To continue our research, a simple and regioselective methodology, by using Eosin Y as a clean photosensitized oxidation catalyst, is developed for the synthesis of peroxide bridge in steroids. The method was exposed to visible light and furnished in high yields, does not involve tedious work-up or purification, and avoided using environmentally hazardous solvents. It could be regarded as a green protocol. Besides, a series of cholesterol and β-sitosterol derivatives containing a peroxide bridge were synthesized through this method and screened for their anti-HBV activity. Among compounds synthesized in this research, 5α, 8α-cyclicobioxygen-6-vinyl-3-oxo-cholesterone (**1f**, 3.13μg/ml) had the most potent activity with inhibition rates of 77.45±6.01% and 58.73±8.64 % on the secretion of HBsAg and HBeAg antigens after 8 days. Further acute toxicity test showed that the LD_{50} value of compound **1f** was 362.46 mg/kg after intraperitoneal injection in mice. Moreover, structure-activity relationships of cholesterol and β-sitosterol derivatives were briefly discussed

Introduction

Steroids, a widespread class of natural organic compounds occurring in animals, plants and fungi, play a highly significant role in the drug discovery [1-3]. These compounds had shown remarkable biological activities, including antiviral, anti-tumor, antihypertensive, anticholinergic and so on [4-8]. In previous work, a new steroid compound (alcohol from ascidian, SC) with peroxide bridge was discovered , which exhibited potent anti-hepatitis B virus activity [9-11]. However, due to the low content of SC in Styela Clava [12], it has stimulated our interest in synthesizing its structural analogs. Cholesterol and β-sitosterol have the same parent nucleus with SC (Figure 1). So they were expected to be anti-HBV drugs by building peroxide bridges.

In our present study, a new method was established to synthesize peroxide bridge by using Eosin Y as photosensitive catalytic oxidizer, which had been patented [13]. The method was operated at room temperature and with visible light, which was simple and environmentally friendly compared with the conventional synthetic methods [14-16]. The experimental results from the synthesis parts indicated that Eosin Y could combine with oxygen in the air and then oxidize dienes into peroxide bridges, which might be regarded as a specific catalytic to diene ring in the 5,8 position of cholesterol and β-sitosterol. Therefore, we will continue to focus on its application in other steroids and optimize chemistry technology for synthesizing peroxide bridge.

In our continuous effort a series of novel cholesterol and β-sitosterol derivatives with peroxide bridges were synthesized through the new method. All newly derivatives were fully characterized with ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectra (HMQC, HMBC and ^{1}H-^{1}H COSY) and HRMS. Their inhibitory effects on the expression of HBsAg and HBeAg in the HepG2.2.15 cells were evaluated by MTS and ELISA assays. Moreover, acute toxic test, as a part of safety evaluation of peroxo-bridged derivatives, was carried out to investigate the potential toxicity by intraperitoneal injection in ICR mice. In addition, the structure-activity relationships of these novel compounds were also briefly discussed.

Results and Discussion

Chemistry

All designed derivatives were synthesized via the routes outlined in Scheme 1. Compounds **1a** and **2a** were prepared via acetylation reaction of cholesterol and β-sitosterol, and then underwent free radical and elimination reaction with N-bromosuccinimide (NBS) to

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afford compounds **1b** and **2b**. Subsequently, products **1c** and **2c** were obtained after deacetylation reaction of **1b** and **2b**. Then **1b** connected with δC 80.0 (C-8), which were similar with **1b**. Besides, there were δC 83.4 (C-5) and δC 80.0 (C-8) in the ^{13}C-NMR spectrum

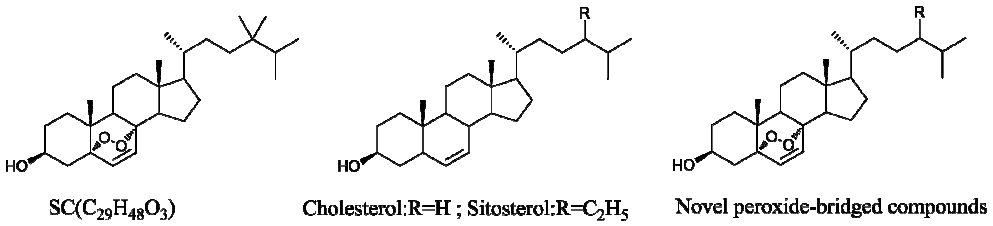
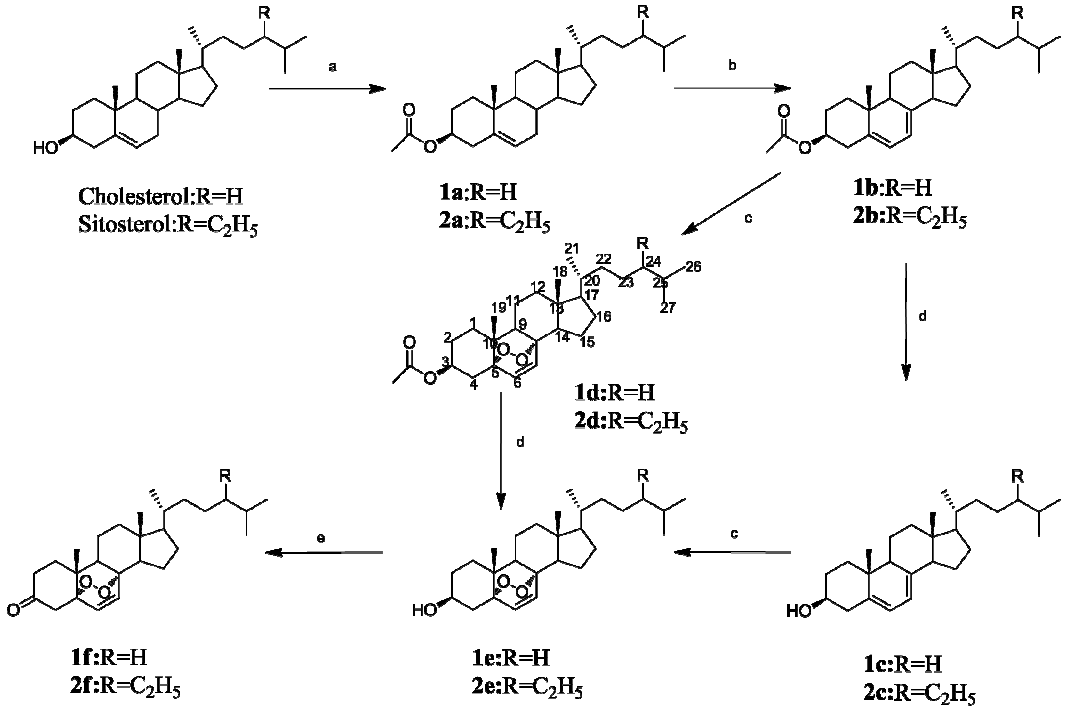


Figure 1 The structures of SC, cholesterol, β-sitosterol and the novel peroxo-bridged derivatives.



Scheme 1 Synthesis of the cholesterol and β-sitosterol derivatives. Reagents and Conditions: (a) Toluene, acetic anhydride, pyridine, reflux, 4 h. (b) CCl_4, N-bromobutanimide (NBS), lighted, reflux, 4 h. (c) Eosin Y, anhydrous ethanol, lighted, r.t., 10 h. (d) Ethanol, 10% sodium hydroxide, 80°C, 30 min. (e) Acetone, chromic acid, ice-bath, 3 h.

and **2b** were further reacted with Eosin Y under visible light to generate the target compounds **1d** and **2d**. Compound **1e** and **2e** were obtained via two different routes (1): a-b-c-d and (2): a-b-d-c with different yield rates. Compounds **1f** and **2f** were synthesized from **1e** and **2e** with chromic acid solution in acetone eventually. All newly derivatives were fully characterized with ^{1}H-NMR, ^{13}C-NMR and 2D-NMR spectra (HMQC, HMBC and ^{1}H-^{1}H COSY) and HRMS.

The molecular formula of **1f** was assigned as $C_{27}H_{42}O_3$ through the basis of the positive high-resolution mass spectrometer (HR-MS) m/z 437.30087 $[M+Na]^+$. Especially, product **1f** was further identified by HMQC, HMBC and ^{1}H-^{1}H COSY 2D-NMR spectra. Combined with the significant HMQC, HMBC and ^{1}H-^{1}H COSY correlations, CH-6 [δH 6.53 (d, 1H, *J* = 8.5 Hz, =CH-)] was connected with δC 83.4 (C-5), and CH-7 [δH 6.27 (d, 1H, *J* = 8.5 Hz, =CH-)] was of compound **1f**, while δC 141.6 (C-5) and δC 138.6 (C-8) in the ^{13}C-NMR spectrum of **1b**. It suggested that the peroxide bridge of compound **1f** was synthesized according to all of the above analysis, This inference could also be applied to compounds **1d** and **1e**.

Within the framework of green chemistry, solvents occupy a strategic place. Green chemistry aims to replace the hazardous and/or harmful solvents with more environmentally friendly alternatives. In our experiment, Eosin Y, a kind of dye [17]was used as a photosensitive catalytic oxidizer[18,19]. It could simplify the use of Eosin Y as a photosensitive catalytic oxidizer simplified the reaction and avoided the use of H_2SO_4, H_2O_2 and the expensive materials: MTO and Re_2O_7 [20, 21], which might be regarded as a relatively green process. In addition, the mechanism involved of the reaction is probably that Eosin Y activated the oxygen in the air, and then the structure of conjugated dienes was peroxidized to obtain corresponding products. What's more, the result of the catalytic

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reactions appears that Eosin Y has specificity for synthesizing peroxide bridge in diene.

Biological activity

The cytotoxicity of all compounds in HepG2.2.15 cells

The cytotoxicity of all compounds was observed by a standard MTS method. After the incubation with various concentrations of these synthetics, the inhibition ratio of the HepG2.2.15 cells exposed to different values of the compounds, as shown in Figure 2. There was a dose-effect relationship in all derivatives on cytotoxicity. These results were used to determine the treatment concentration of these six compounds for the subsequent experiments.

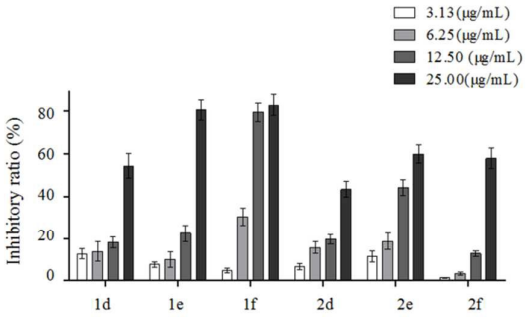

Figure 2 Cytotoxicity effect of **1d-1f** and **2d-2f** on HepG2.2.15 cells line.

Inhibition of HBsAg and HBeAg production in HepG2.2.15 cells

As shown in Table 1, all derivatives with peroxide bridge exhibited higher activity on anti-HBV than the others. Current study proved that introducing peroxide bridge to SC parent steroid nucleus could induce the anti-hepatitis B virus activity. Furthermore, it also proved that various 3-substituents could influence peroxo-bridged derivatives' biological activity.

Table 1 Inhibitory effect of **1d-1f** and **2d-2f** on HBsAg and HBeAg secretion in HepG2.2.1 cells at 3.13 μg/mL

Compound	4 d		8 d	
	HBsAg (%)	HBeAg (%)	HBsAg (%)	HBeAg (%)
1d	12.96±3.45	3.51±2.65	13.77±4.23	7.56±4.78
1e	1.20±4.67	1.35±4.87	1.56±3.12	2.55±5.01
1f	72.28±6.00	54.10±5.06	77.45±6.01	58.73±8.64
2d	26.67±2.98	25.64±3.23	28.79±4.34	25.78±3.54
2e	19.01±2.65	2.88±5.45	20.04±3.12	10.35±3.56
2f	1.91±4.65	3.28±4.98	5.70±4.13	2.36±5.37
3TC	5.68±3.78	4.94±4.99	-0.44±3.85	-9.22±6.32

For example, activity of **1f** was more remarkable than **1d** and **1e**, which suggested 3-carbonyl might promote the anti-HBV activity of cholesterol derivatives with peroxide bridge. While, compound **2d** exhibited stronger activity than **2e** and **2f**, which demonstrated 3-acetyl in β-sitosterol derivatives may play a key role. It thus appeared that diverse sterol matrixes had different working groups. The phenomenon could be related to their biogenetic derivation.

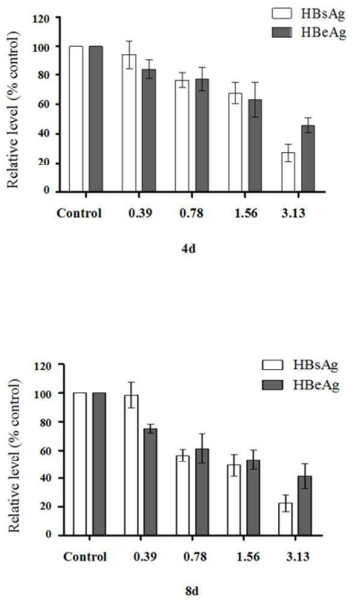

Figure 3 The inhibition ratio of compound **1f** on HBsAg and HBeAg in HepG2.2.15 cells.The inhibition ratio of compound **1f** on HBsAg and HBeAg in HepG2.2.15 cells. HepG2.2.15 cells were treated with four concentrations (0.39, 0.78, 1.56, and 3.13 μg/ml) of compound **1f** for 4d or 8d. The cultural media of each treatment were collected for viral HBsAg and HBeAg via ELISA analysis. The data are expressed as the mean and the standard deviation of the mean. (n = 3, $p<0.05$).

Further study on antigens inhibition of compound **1f** was studied by setting four concentration gradients. As shown in Figure 3, dose- and time-effect relationship was found, and after 8 days, **1f** showed significant inhibitory effects on HBsAg secretion, with the highest inhibition at 77.45±6.01%. Similarly, the secretion of HBeAg was significantly reduced with the highest inhibition at 58.73±8.64%.

Meanwhile, we calculated the therapeutic index of **1f** to indicate its activity of anti-hepatitis B (Table 2).

Table 2 Inhibitory effect on HBsAg and HBeAg in 2.2.15 cells treated with **1f**

Time	Antigen	IC_{50}	TC_{50}	TC_{50}/IC_{50}
4d	HBeAg	1.78±0.24	10.25	5.79±0.52
	HBsAg	1.86±0.09		5.51±0.18
8d	HBeAg	2.59±0.15		3.40±0.24
	HBsAg	2.95±0.35		3.47±0.37

Acute toxicity of compound 1f in *vivo*

Although, compound **1f** exhibited relatively low cytotoxicity in *vitro*, the toxicity in *vivo* was not clear. To evaluate the safety of compound **1f**, the acute toxicity was tested in ICR mice. At dose of

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56, 178, 350, 422, 750 and 1000 mg/kg administered intraperitoneally (i.p.). Every dose was given 6 mice, compound **1f** revealed a regular dose-dependent increase in mortality (Table 3).

The LD_{50} value (362.46mg/kg) can be worked out through the regression equation: y (Probit) = -17.114 + 8.6408 Log (D), which showed the safe dose range of **1f.**

(Statement: All experiments were performed in compliance with the relevant laws and institutional guidelines, and the institutional committee(s) that have approved the experiments)

Table 3 Acute toxicity test of compound **1f**

Dose (mg/kg)	Mice Number Start/End	Death rate (%)	LD50 (mg/kg)	95%CIs
1000	6/6	100	362.46	233.83-561.85
750	6/6	100		
422	6/4	67		
350	6/3	50		
178	6/0	0		
56	6/0	0		

Conclusion

A new method to synthesize peroxo bridge was established by using Eosin Y as photosensitive catalytic oxidizer. Comparedwith the conventional synthetic methods, it was in lower consumption and more convenient. Therefore, our synthesis of peroxide bridges had been optimized by the special catalyzer. A series of novel steroid derivatives containing peroxide bridge were synthesized using this method, and their bioactivity tests showed that most derivatives had anti-HBV activities. In particularly, compound **1f** exhibited higher inhibitory against HBsAg and HBeAg secretion in HepG2.2.15 cells, while nearly no cytotoxicity on HepG2.2.15 cells at 3.13 μg/mL, showing good therapeutic index.

The structures were significantly different between steroid derivatives and 3TC. Although, lamivudine was no longer recommended as first-line therapy for chronic hepatitis B, and using lamivudine judiciously on the clinical and scientific research was paramount [22]. Unlike the metabolism antagonism of 3TC, steroids might indicate yet another mechanism that is related with the capacity of steroid hormones to intercalate into cell membrane bilayer and alter membrane fluidity [23]. This property of the steroids would affect the insertion of viral glycoproteins further inhibiting the assembly of virus particles [4], which was pending further study. Therefore, these compounds were expected in collaboration with 3TC for the treatment of hepatitis B diseases. In summary, the steroid derivatives with peroxide bridges demonstrated potential anti-HBV activity, and it provided us new ideas to discover anti-HBV leading compounds.

Acknowledgments

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