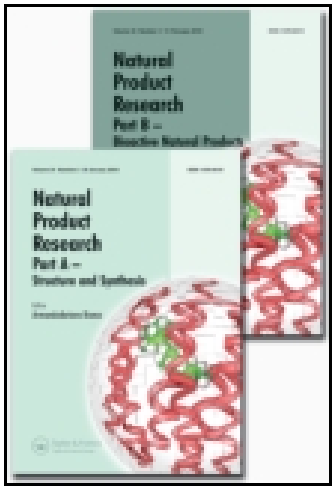


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# The semisynthetic spin-labelled derivatives of 3-hydroxybutanolide as potential oxidative stress inhibitors

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To obtain more accessible oxidative stress inhibitors, a series of novel spin-labelled derivatives of 3-hydroxybutanolide (**2a**–**d**, **3a**–**d**) with the natural active compound (kinsenoside) as the lead compound were designed, synthesised from the nitroxide free radical piperidine (pyrroline) and the main structural unit of kinsenoside: 3-hydroxybutanolide. Antioxidant activity screening of these derivatives was performed using MTT method on rat pheochromocytoma PC12 cells. The antioxidative stress effect was further investigated on the changes of the important antioxidant enzyme activities and intracellular reactive oxygen species production. Among the derivatives, **2b**–**d**, **3a** and **3c** showed comparable or superior antioxidative stress activity to kinsenoside. Also, most of the tested derivatives displayed obvious antioxidative ability in concentrations. Cytotoxic assay simultaneously indicated that all compounds had very low toxicity to normal cells. Based on the observed results, the structure–activity relationship of these derivatives was discussed.

**Keywords:** 3-hydroxybutanolide; spin-labelled; antioxidant activity; synthesis



## 1. Introduction

There are many derivatives of 3-hydroxybutanolide existing in nature. Kinsenoside is a classic one. It is 3-(*R*)-3-β-D-glucopyranosyloxybutanolide (Figure 1) and a major ingredient of *Anoectochilus roxburghii* which is one rare species belonging to the genus *Anoectochilus* (Orchidaceae). *A. roxburghii* is a Chinese folk medicine used to treat diabetes (Zhang et al. 2007), cancers (Tseng et al. 2006), liver diseases (Shih et al. 2004), cardiovascular diseases (Liu et al. 2013), etc. *A. roxburghii* is also made into popular nutraceutical healthcare products in China and other Asian countries because of its healthcare effects. Recently, kinsenoside exhibited significantly antihyperglycaemic activity and possessed antioxidant, anti-inflammatory, immunostimulating and hepatoprotective activities (Wu et al. 2007; Du et al. 2008; Hsiao et al. 2013). Zhang et al. (2007) reported that the antihyperglycaemic effect of kinsenoside might be attributed to modulating the activity of enzymatic antioxidants, scavenging free radicals and reducing the content of factor NO in diabetic rats. In our previous study, kinsenoside showed significant vascular protective effects in *in vitro* and *in vivo* experiments. And this effect of kinsenoside was speculated due to the oxidative stress inhibition and the reduction of nuclear factor kappa B (NF-κB) mRNA expression levels in high glucose conditions (Liu et al. 2013).

Researches have revealed that oxidative stress was caused by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify

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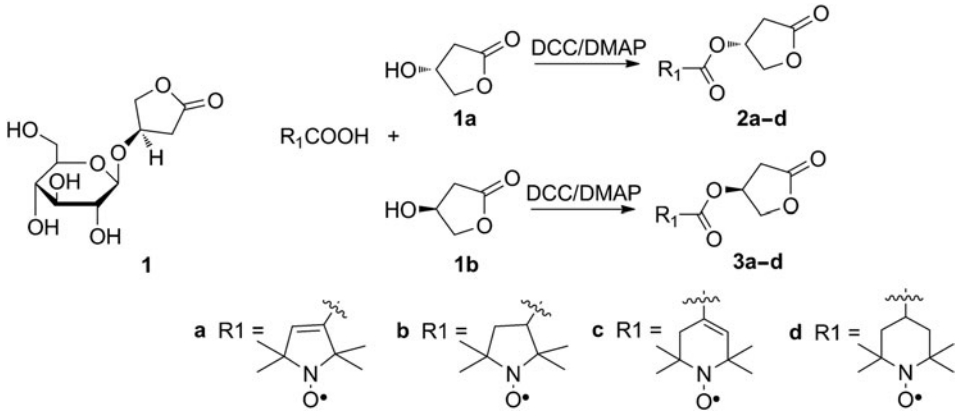


Figure 1. The structure of kinsenoside (**1**) and the synthesis of compounds **2a**–**d** and **3a**–**d**.



the reactive intermediates or to repair the resulting damage. It is thought that oxidative stress was involved in the progression of pathogenesis of diabetes, atheromatous plaque, stroke, Alzheimer's disease, Parkinson's disease, etc. (Dewapriya et al. 2013).

Oxidative stress resistance strategy is used to treat the diseases related to oxidative stress and proved to be a practicable treatment based on the root cause of diseases. Antioxidant stress drugs are the key to oxidative stress resistance strategy. Many known antioxidants, such as ferulic acid and quercetin (Hsieh et al. 2010), cannot be ideal antioxidant stress drugs because of their prooxidant side effects on normal cells or being synergies with other agentia, such as vitamin C or glutathione. The discovery of a new antioxidant and development of excellent antioxidant stress drugs have still attracted the interest of researchers. Kinsenoside is a good potential oxidative stress inhibitor (Du et al. 2008). Our results also showed that kinsenoside was a good antioxidant lead. In the study, the protective effect of kinsenoside against $H_2O_2$-induced oxidative damage in PC12 cells was obvious (Table 2) and the toxicity of kinsenoside to normal PC12 cells was quite low (Table S1). But kinsenoside is not easily available because of scarce natural resources and artificially synthesised approach which is a multi-step process or difficult in separating (Zhang et al. 2005). A series of derivatives with kinsenoside as the lead compound were therefore designed to obtain more accessible antioxidants (Figure 1 and Table 1).

(*R*)-3-Hydroxybutyrolactone is the main structural unit of kinsenoside and might play an important role for biological function of kinsenoside. The stable nitroxides are the antioxidants which can provide cytoprotection in mammalian cells against diverse types of oxidative insult and identify structural determinants optimal for protection against individual types of damage. Synthesis of stable nitroxyl radicals spin-labelled bioactive molecules was proved to be an efficient method to obtain the effective therapeutic agents with high activities and less toxicity to normal cells (Hendricks et al. 2012). Herein, we continue the study of our group on the discovery and development of bioactive spin-labelled molecules derived from natural products (Liu et al. 2012). Stable nitroxyl radicals spin-labelled derivatives of 3-hydroxybutanolide (**2a**–**d**) and the corresponding epimers (**3a**–**d**) were synthesised and biologically evaluated because the configuration at 3-position was simultaneously considered as another important factor affecting bioactivities.

## 2. Results and discussion

The new compounds (**2a**–**d**, **3a**–**d**) synthesised (Figure 1, Table 1) were screened for antioxidant activity through the suppression test on the $H_2O_2$-induced oxidative stress damage in

Table 1. Physical and spectroscopy characters of compounds **2a–d** and **3a–d**.

| | | | | HR-MS (ESI) | | | ESR | | |
|---|---|---|---|---|---|---|---|---|---|
| Compounds | Molecular formula | M·p·(°C) | $[\alpha]_D^{20}$ ($c = 0.5$, $CH_3COCH_3$) | Calculated | Found | IR | $g$ | $a_N$ (G) | $\triangle H$(G) |
| **2a** | $C_{13}H_{18}O_5N$ | 121–123 | 68.238 | 286.1523 $[M + NH_4]^+$ | 286.1527 $[M + NH_4]^+$ | 1357(N—O˙) 1785(C═O) | 2.0064 | 15.14 | 1.27 |
| **2b** | $C_{13}H_{20}O_5N$ | | 50.831 | 288.1680 $[M + NH_4]^+$ | 288.1686 $[M + NH_4]^+$ | 1369(N—O˙) 1787(C═O) | 2.0064 | 16.17 | 1.30 |
| **2c** | $C_{14}H_{20}O_5N$ | 131–132 | 19.053 | 300.1680 $[M + NH_4]^+$ | 300.1673 $[M + NH_4]^+$ | 1356(N—O˙) 1783(C═O) | 2.0063 | 15.61 | 1.13 |
| **2d** | $C_{14}H_{22}O_5N$ | 142–144 | 63.000 | 302.1836 $[M + NH_4]^+$ | 302.1830 $[M + NH_4]^+$ | 1361(N—O˙) 1781(C═O) | 2.0064 | 16.14 | 1.60 |
| **3a** | $C_{13}H_{18}O_5N$ | | −58.932 | 286.1523 $[M + NH_4]^+$ | 286.1525 $[M + NH_4]^+$ | 1357(N—O˙) 1785(C═O) | 2.0064 | 15.14 | 1.27 |
| **3b** | $C_{13}H_{20}O_5N$ | | −51.5523 | 288.1680 $[M + NH_4]^+$ | 288.1686 $[M + NH_4]^+$ | 1369(N—O˙) 1786(C═O) | 2.0064 | 16.17 | 1.29 |
| **3c** | $C_{14}H_{20}O_5N$ | 113–115 | −57.803 | 300.1680 $[M + NH_4]^+$ | 300.1671 $[M + NH_4]^+$ | 1357(N—O˙) 1782(C═O) | 2.0063 | 15.61 | 1.13 |
| **3d** | $C_{14}H_{22}O_5N$ | 118–120 | −14.286 | 302.1836 $[M + NH_4]^+$ | 302.1830 $[M + NH_4]^+$ | 1361(N—O˙) 1782(C═O) | 2.0064 | 16.14 | 1.60 |

PC12 cells by the MTT method. As shown in Table 2, all compounds exhibited *in vitro* antioxidant activity within the experimental dose range (10–90 μg/mL). Particularly, like kinsenoside, compounds **2b–d**, **3a** and **3c** remarkably protected PC12 cells against $H_2O_2$-induced cytotoxicity on different doses and simultaneously had very low toxicity to normal cells in the cytotoxicity assay (Table S1). The results showed that the replacement of 3-glycosyl group in the kinsenoside structure with nitroxide radical could bring about comparable or superior antioxidants to kinsenoside.

The antioxidative stress ability of the molecules was further studied. The effects of **2a–d**, **3a–d** on up-regulating the activities of the two important antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and down-regulating intracellular ROS production were detected in injured PC12 cells treated with $H_2O_2$ (Figure 2). As seen in Figure 2(A)–(C), SOD seemed sensitive to all compounds although the activity of CAT substantially had consistent change trend with that of SOD. Meanwhile, the intracellular ROS production showed a contrary tendency with the changes of the antioxidant enzyme activity after the cells were pretreated with the compounds. Among the compounds, **2b–d**, **3a** and **3c** increased the antioxidant enzyme activity and inhibited intracellular ROS generation significantly.

Based on these results, some preliminary structure–activity relationships (SAR) on these derivatives were then investigated. For the derivatives (**2a–d**) with 3*R*-configuration, they generally emerges higher antioxidative ability than the derivatives (**3a–d**) with 3*S*-configuration in no matter screening test or the enzyme activity assay, or even in the intracellular ROS detection. This phenomenon is analogical to the phenomenon that kinsenoside has higher anti-hyperliposis effect than its epimer: (3*S*)-3-β-D-glucopyranosyloxy (butanolide, goodyeroside A) (Du et al. 2008). A possible explanation for this phenomenon is that configuration affects the affinity between drug molecules and receptor. This phenomenon also provides an evidence for (*R*)-3-hydroxybutyrolactone as the main active structural unit of kinsenoside. In addition, in the experiments of this study, the activities of derivatives of (*R*)-3-hydroxybutanolide with saturated pyrroline (piperidine) (**2b** and **2d**) were stronger than those of derivatives with unsaturated pyrroline (piperidine) (**2a** and **2c**). But the reverse happened in the derivatives of (*S*)-3-hydroxybutanolide with piperidine and piperidine (**3a** > **3b**, **3c** > **3d**). The law and the mechanism are a subject of further investigations.

In summary, a series of novel spin-labelled derivatives of 3-hydroxybutanolide with kinsenoside as the lead compound were designed, synthesised and evaluated to develop more potential oxidative stress inhibitors. Some derivatives (**2b–d**, **3a** and **3c**) showed very impressive antioxidative stress activity and the 3-nitroxide radical derivative was demonstrated an effective method to obtain potential oxidative stress inhibitors. The different antioxidative activity of these compounds indicated that the 3-configuration of kinsenoside structure markedly affected the activity profiles of this compound class, and the important SAR information has been revealed. It will pave the way for the design and development of derivatives of 3-hydroxybutanolide as potential oxidative stress inhibitors in the future.

## 3. Experimental

### 3.1. *Materials and general methods (See supplementary material)*

### 3.2. *Synthesis*

The nitroxide free radical piperidine (pyrroline) was synthesised according to the literature (Elmer et al. 1976). As described in Figure 1, under argon atmosphere, (*R*)-3-hydroxybutyrolactone or (*S*)-3-hydroxybutyrolactone (102 mg, 1.0 mmol) and nitroxide free radical piperidine (pyrroline) (1.0 mmol) were dissolved in dry dichloromethane (DCM) (20 mL). Then 4-dimethylaminopyridine (24 mg, 0.2 mmol) was added into the above solution. After the mixture was stirred for 5 min at room temperature, dicyclohexyl carbodiimine (206 mg,

Table 2. PC12 cell viability under concentrations from 10 to 90 μg/mL of the derivatives (**2a**–**d** and **3a**–**d**) of kinsenoside (kin for short).

| Compound | Control | Model group | Test groups with different dosages | | | | |
|---|---|---|---|---|---|---|---|
| | | | 10 (μg/mL) | 30 (μg/mL) | 50 (μg/mL) | 70 (μg/mL) | 90 (μg/mL) |
| Kin | 100 ± 0.00 | 59.54 ± 4.78** | 67.94 ± 4.78$^{**\#}$ | 67.55 ± 8.60$^{**\#}$ | 67.78 ± 2.99$^{**\#}$ | 67.69 ± 7.37$^{**\#}$ | 50.15 ± 4.57$^{**\#}$ |
| **2a** | | 39.65 ± 2.53** | 45.75 ± 7.98** | 52.67 ± 5.21$^{**\#\#}$ | 45.49 ± 6.74** | 47.56 ± 5.87** | 48.29 ± 9.36** |
| **3a** | | 47.50 ± 4.76** | 59.16 ± 3.96$^{**\#\#}$ | 55.61 ± 5.82$^{**\#\#}$ | 64.06 ± 5.58$^{**\#\#}$ | 56.89 ± 4.37$^{**\#\#}$ | 51.39 ± 3.17** |
| **2b** | | 41.56 ± 3.14** | 47.47 ± 3.79** | 45.97 ± 5.24** | 54.67 ± 8.13$^{**\#\#}$ | 56.29 ± 6.37$^{**\#\#}$ | 56.88 ± 11.17$^{**\#\#}$ |
| **3b** | | 63.58 ± 5.14** | 60.97 ± 4.94** | 65.12 ± 10.84** | 71.19 ± 11.69** | 74.02 ± 7.22$^{**\#}$ | 62.02 ± 8.44** |
| **2c** | | 49.73 ± 9.38** | 50.91 ± 9.80** | 68.07 ± 10.80$^{**\#}$ | 56.57 ± 11.53** | 70.06 ± 8.05$^{**\#\#}$ | 63.67 ± 7.47$^{**\#}$ |
| **3c** | | 76.07 ± 7.39** | 88.99 ± 6.13$^{**\#\#}$ | 89.85 ± 5.92$^{**\#\#}$ | 87.35 ± 5.90$^{**\#\#}$ | 88.15 ± 7.35$^{**\#\#}$ | 86.06 ± 3.76$^{**\#}$ |
| **2d** | | 50.12 ± 8.76** | 49.02 ± 10.12** | 47.30 ± 7.90** | 42.54 ± 7.09** | 65.26 ± 6.58$^{**\#\#}$ | 61.83 ± 7.14$^{**\#}$ |
| **3d** | | 52.43 ± 2.32** | 56.47 ± 6.43** | 54.68 ± 3.06** | 52.51 ± 2.70** | 54.05 ± 3.72** | 56.05 ± 3.76** |

Notes: Data are expressed as a percentage of control and mean ± SD from six experiments. A value of $P < 0.05$ was considered to be statistically different. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus model group.

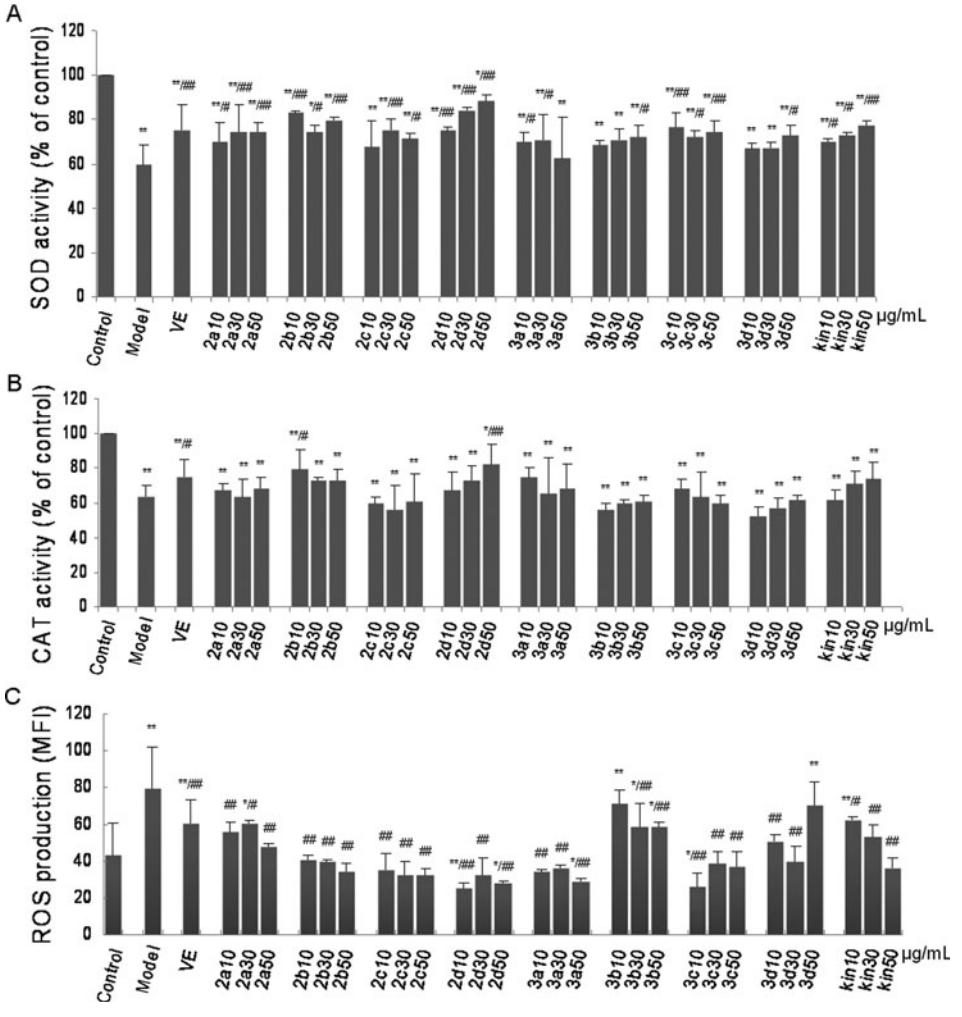


Figure 2. Effects of compounds on the activities of SOD, CAT and ROS production on $H_2O_2$-injured PC12 cells. Data are mean ± SD from three experiments. A value of $P < 0.05$ was considered to be statistically difference. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus model group.

1.0 mmol) was successively added. The reaction mixture was stirred for another 2 h until TLC revealed the absence of the starting material. The mixture was filtered and the filtrate was concentrated in vacuum. The residue was purified by column chromatography on silica gel using DCM/acetone (15/1) as eluent to give compounds **2a**–**d**, **3a**–**d**.

### 3.3. *Physical and spectroscopy characters of kinsenoside and compounds 2a–d and 3a–d (See supplementary material and Table 1)*

### 3.4. In vitro *antioxidant activity assay*

#### 3.4.1. *Cell culture*

PC12 cells were seeded onto 96-well plates at a density of $1.0 \times 10^5$ cells/mL in Dulbecco's Modified Eagle's Medium, supplemented with 10% new calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37°C under a 5% $CO_2$ humidified atmosphere.

3.4.2. *Effect of the derivatives on cells viability*

To assess the cytotoxicity of the derivatives of 3-hydroxybutanolide (10–90 μg/mL), the cells were incubated in MTT solution (0.5 mg/mL) in phosphate-buffered saline (PBS) for 4 h. The formation of purple formazan was quantified by measuring the absorbance at a wavelength of 570 nm with background subtraction using a wavelength of 630 nm on a microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA)

3.4.3. *The cell protection assay*

To test the protective effects of kinsenoside and the derivatives in PC12 cells, the proliferative activity was evaluated (Li et al. 2003). Hydrogen peroxide ($H_2O_2$) was stored at 4°C, and 100 mM stock solution was freshly prepared in PBS before being applied to the cultures with a final concentration of 200 μM. The PC12 cells were pretreated with different derivatives for 1 h, and then the cells were incubated with $H_2O_2$ for 24 h. Meanwhile, the test groups were divided into the control (standard growth medium), the model (high $H_2O_2$), vitamin E (800 μM) treatment and the derivative (10, 30 and 50 μg/mL) treatment groups. Cell survival was evaluated by the colorimetric assay of MTT to test the protection.

3.4.4. *Antioxidant assay*

To evaluate the antioxidant effects of the derivatives, the activities of SOD and CAT and the ROS production were examined.

Intracellular ROS production was measured by using a fluorescent dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Zhao et al. 2011). Briefly, cells were seeded into 24-well plates and pretreated with derivatives for 1 h, and then the cells were incubated with $H_2O_2$ for 24 h. The treated cells were loaded with 10 μM DCFH-DA in the medium for 30 min. After washing three times with PBS, the cells were digested with 0.25% trypsin. The digestive cells were collected and transferred into EP tube. Then, the single cell suspension was made. The fluorescence was analysed by flow cytometry through the FL1 channel. ROS levels are expressed as mean fluorescence intensity.

3.4.5. *Statistical analysis*

All data are presented as means ± SD for three or six independent experiments. Statistical analysis was performed by one-way ANOVA followed by LSD test. A *P*-value of less than 0.05 was considered to be statistically significant.

## 4. Conclusion

Our results have not only enriched the family of derivatives of 3-hydroxybutanolide, but have also encouraged the synthesis of spin-labelled derivatives for improving antioxidant activity.

## Supplementary material

Experimental details relating to this article are available online, alongside Table S1.

## Acknowledgements

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