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Synthesis, immunological activities, and scavenging ability toward superoxide anion of (1→3)-β-D-pentaglucoside and its epoxyalkyl derivatives

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Abstract—The epoxyalkyl (**1**→**3**)-β-D-pentaglucosides **2** and **3** were synthesized in order by acetylation, glycosidation, oxidation, and deacetylation of **1**. The immunological activities (superoxide anion production activity, phagocytic activity, and lymphocyte proliferation) and scavenging ability toward superoxide anion of (1→3)-β-D-pentaglucoside (**1**) and its epoxyalkyl derivatives (**2** and **3**) were investigated. Superoxide anion released from human blood monocytes was measured by the reduction of ferricytochrome *c*. Phagocytosis by peritoneal macrophages was detected through a teal ingesting that measured the chicken red blood cells (CRBC). Lymphocyte proliferation was determined by the MTT method. The scavenging ability of **1**, **2**, and **3** toward superoxide anions was evaluated by means of chemiluminescence (CL). The results showed that **2** and **3** had a little higher immunological activity and scavenging ability toward superoxide anion than **1**, which indicated that the reducing end of the oligoglucosides was quite important for maximum biological activity.

1. Introduction

In the last years, increased attention has been paid to various types of immunomodulating fungal (1→3)-β-D-glucans studied that soluble glucans having (1→3)-β-glucosyl linkages have good activity in promoting biological events, such as phagocytosis, hydrogen peroxide synthesis, and cytokine synthesis.[1] Moreover, it is interesting that a specific hydrolase to degrade β-glucans has been identified neither from men nor mice. Thus, β-glucans once deposited in the body remain for very long periods of time without major structural changes.[2,3] However, much controversy surrounds the biochemical and molecular principles of the immunostimulatory activity of β-glucans. In any case, the expression of the immunological activity presupposes certain interactions between the glucans and the macrophages or other cells of the biological systems studied. These interactions are dependent on the structural and molecular parameters of the soluble β-glucans mentioned earlier.

'Reactive oxygen species' (ROS) is a collective name for a group of oxygen-containing species such as superoxide anion, hydroxyl radical, and hydrogen peroxide. ROS are widely considered to induce cancer,[4] aging,[5] and some other chronic diseases.[6] It has been demonstrated that some oligosaccharides have the scavenging effects on ROS.[7] We know that electron spin resonance (ESR) and chemiluminescence (CL)[8] can be used to measure the scavenging ability toward ROS of oligosaccharides. However, CL is a simple, direct, and effective method for ROS and antioxidant studies, which has been proven to be a sensitive assay for tracing the reaction process of antioxidant scavenging of ROS. In this paper, the scavenging ability of **1**, **2**, and **3** toward superoxide anions is measured by means of CL.

The use of epoxyalkyl compound as active-site-directed inhibitors has been invaluable in delineating

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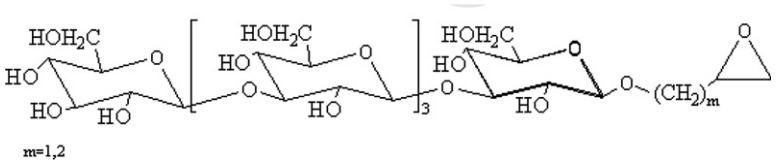


Scheme 1. Epoxyalkyl (1→3)-β-D-pentaglucosides **2** (m = 1) and **3** (m = 2).

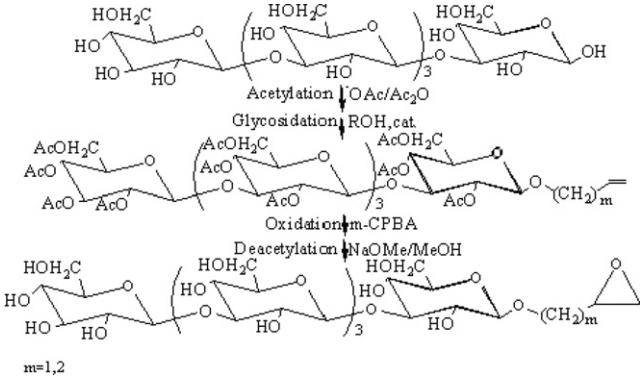
the mechanism of action for a variety of hydrolases, for example, β-D-glucan endo- and exohydrolases.[9,10] At the same time, the oxygen function can be introduced as an epoxide, and such a modification could provide additional information regarding the structure–function relationships of oligo-(1→3)-β-D-glucosides. We therefore synthesized and analyzed **1**, **2**, and **3** with epoxy structures in the aglycone (Scheme 1).[11–13] The aim of the present work was to find out whether their immunomodulating properties and scavenging ability toward superoxide anion had been affected by the epoxyalkyl derivatization of **1**.

2. Results and discussion

Some oligosaccharides seem to enhance the activity of the immune system, but today there is no accepted mechanism, and not even any agreement on the parameters, which influence the activity. To study the effects of the structure on the immunological activity of **1**, **2**, and **3**, three laboratory tests were used: superoxide anion production activity, phagocytosis by peritoneal macrophages, and lymphocyte proliferation. At the same time, we also measured the scavenging ability toward superoxide anion of **1**, **2**, and **3**.

2.1. Synthesis of epoxyalkyl (1→3)-β-D-pentaglucosides 2 and 3

(1→3)-β-D-Pentaglucoside **1** was acetylated with potassium acetate-acetic anhydride to maximize the yield of peracetylated (1→3)-β-D-pentaglucoside. The peracetylated (1→3)-β-D-pentaglucoside was treated with allyl alcohol and 3-butenyl alcohol and stannic chloride as a Lewis acid catalyst. The reaction of peracetylated allyl and 3-butenyl (1→3)-β-D-pentaglucosides with *m*-CPBA in dichloromethane at room temperature gave the corresponding oxiranes. NaOMe in dry methanol at room temperature was used for the deacetylation of the blocked derivatives, to give the corresponding 2,3-epoxypropyl and 3,4-epoxybutyl (1→3)-β-D-pentaglucosides **2** and **3** in overall yields of 28% and 21%, respectively (Scheme 2). Epoxidation of peracetylated allyl and 3-butenyl (1→3)-β-D-pentaglucosides introduced new chiral centers at C-2 and C-3 of the aglycones, respectively. In the case, the major isomers were isolated and purified by column chromatography on silica gel, and the ^{1}H NMR spectra indicated that C-2 and C-3 of the aglycones were *R* configuration (*R*:*S* = 5:1). It indicated that the anomeric proton of these major compounds **2** and **3** (C-2, C-3 *R*) resonates at higher field (C-2 δ = 3.09, C-3 δ = 3.06) than the minor diastereoisomers (C-2 *S* δ = 3.24, C-3 *S* δ = 3.20).



Scheme 2. Synthetic route of epoxyalkyl (1→3)-β-D-pentaglucosides **2** (m = 1) and **3** (m = 2).

2.2. Superoxide anion production activity

Incomplete reduced forms of oxygen, including superoxide, can play a major role in anti-microbial,[14] antitumor,[15] and inflammatory functions of monocytes,[16] too. The primary reaction is the one-electron reduction of oxygen to $O_2^{\bullet-}$ through the action of a cyanide-resistant NADPH oxidase. The results of experiments with monocytes treated with **1**, **2**, and **3** showed a dependence of 'oxidative' or 'respiratory' burst upon the structure of oligoglucosides. Derivative, **2** and **3** stimulated superoxide production little more than did **1** (Table 1).

2.3. Effect of (1→3)-β-D-pentaglucoside (1) and its epoxyalkyl derivatives (2 and 3) on the phagocytosis by peritoneal macrophages

Table 2 shows the phagocytic activity of macrophages isolated from mice treated with **1**, **2**, or **3**. Our results showed that **1**, **2**, and **3** enhanced the phagocytosis by peritoneal macrophages. Compared to **1**, the phagocytic rate (PR) and the phagocytic index (PI) were elevated a little by treatment with **2** and **3**.

Table 1. Superoxide anion production by human blood monocytes cultured with **1**, **2**, and **3** samples (concentration of **1**, **2**, or **3**, 20 μg/mL; $\Delta O_2^{\bullet-} = O_2^{\bullet-}{}_{1,2\text{ or }3} - O_2^{\bullet-}{}_{\text{control}}$)

Sample	$O_2^{\bullet-}$ production (nmol/10^5/cells)
1	2.9 ± 0.04
2	3.0 ± 0.07[a]
3	3.3 ± 0.11[a]

[a] p <0.05 compared with **1** group.

Table 2. Effects of **1**, **2**, and **3** on the phagocytic rate and phagocytic index of phagocytes from C57BL/6 mice treated everyday for 14 days

Group	Dose (μg/mL)	Mouse no.	Phagocytic rate (PR)	Phagocytic index (PI)
Control	0	4	25.98 ± 2.09	1.04 ± 0.00
1	200	8	55.31 ± 2.37[a]	1.19 ± 0.075[b]
2	200	8	60.78 ± 2.16[a]	1.20 ± 0.080[b]
3	200	8	64.26 ± 2.01[a]	1.23 ± 0.099[b]

[a] p <0.001 compared with control group.
[b] p <0.05 compared with control group.

2.4. Effect of (1→3)-β-D-pentaglucoside (1) and its epoxyalkyl derivatives (2 and 3) on lymphocyte proliferation

To determine the effects of **1**, **2**, and **3** on lymphocyte proliferation, mitogen-induced proliferation of C57BL/6 mice splenocyte was measured by the MTT method.[17] As shown in Figure 1, the average absorbances of lymphocytes from **1**, **2**, and **3** were higher than that to the control, and the average absorbances of **2** and **3** were a little higher than that of **1**.

2.5. Scavenging ability toward superoxide anion

Using the chemiluminescence system of Me_2SO–NaOH–O_2–luminol[18] under optimum luminance conditions of pH = 8.6, the $O_2^{\cdot -}$ was measured. The changes of CL intensity in **1**, **2**, and **3** with time are shown in Table 3. We found out that a marked decrease of CL intensity was observed when **1**, **2**, or **3** was added. The area of CL also denotes the amounts of $O_2^{\cdot -}$. This indicates that the compounds are acting in an antioxidant manner. The results also showed that the scavenging ability toward superoxide anion of **2** and **3** was little higher than that of **1**.

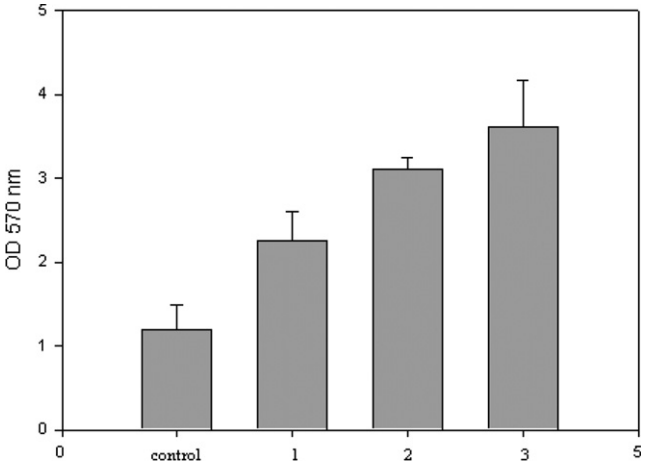


Figure 1. Effects of 14-day treatment of **1**, **2**, and **3** on the proliferation of lymphocytes. Control group mice were treated with normal saline and the dose group mice were treated with 200 μg/mL/day of **1**, **2**, or **3**. The proliferation of splenic lymphocytes was assayed by the MTT method. The difference between the control group and treated groups was determined by Student's t-test. Data are expressed as mean ± SD. The statistical difference existed between **1**, **2**, or **3** group and untreated control group (p <0.05).

Table 3. Effects of compound on scavenging $O_2^{\cdot -}$

Substance	c (μg/mL)	Area of CL	Scavenging ratio (%)
Control	0	38,547	0
1	0.1	14,278	63
2	0.1	8200	78[a]
3	0.1	7970	79[a]

[a] p <0.001 compared with control group.

3. Conclusions

In this study, the results show that the epoxyalkyl (1→3)-β-D-pentaglucosides **2** and **3** had higher immunological activities and scavenging ability toward superoxide anion than (1→3)-β-D-pentaglucoside (**1**), which indicated that the reducing end of the oligoglucosides was quite important for maximum biological activity.

4. Experimental

4.1. General methods

Optical rotations were determined at 25 °C with a Perkin–Elmer Model 241-Mc automatic polarimeter. ^1H and ^{13}C NMR spectra were recorded according to the new IUPAC rules with Bruker ARX 400 spectrometers (400 MHz for ^1H, 75 MHz for ^{13}C) at 25 °C for solns in $CDCl_3$ as indicated. Mass spectra were recorded with a VG PLATFORM mass spectrometer using the ESI mode. Laminaripentaose, namely (1→3)-β-D-pentaglucoside, was purchased from Sigma–Aldrich Chemical Company. Dichloromethane and 1,2-dichloroethane were distilled from P_2O_5.

4.2. Synthesis of epoxyalkyl (1→3)-β-D-pentaglucosides (2 and 3)

Compounds **2** and **3** were prepared following the procedure described by Huang et al.[11–13] Peracetylated allyl and 3-butenyl (1→3)-β-D-pentaglucosides were synthesized by treating peracetylated (1→3)-β-D-pentaglucoside with the allyl alcohol and 3-butenyl alcohol and Lewis acid (stannic chloride) catalyst, respectively. Epoxidation of the peracetylated allyl and 3-butenyl pentaglucosides took place by *m*-chloroperoxybenzoic acid (*m*-CPBA). NaOMe in dry MeOH was used for the deacetylation of the protected derivatives, to give the 2,3-epoxypropyl and 3,4-epoxybutyl (1→3)-β-D-pentaglucosides **2** and **3**, respectively. Compound **2**: $[\alpha]_D$ +86.3 (c 1.1, $CHCl_3$); ^1H NMR (D_2O, 400 MHz): δ 5.24 (1H, d, $J_{1,2}$ 3.5 Hz, H-1′), 4.62–4.54 (5H, m, H-$1^{V,\ IV,\ III,\ II}$), 3.92–3.85 (5H, m), 3.75–3.33 (19H, m), 3.25–3.19 (6H, m), 3.24 (1H, m, –$CH_2CH(O)CH_2$, *S*), 3.09 (1H, m, –$CH_2CH(O)CH_2$, *R*), 2.76–2.68 (2H, m, –$CH_2CH(O)CH_2$); ^{13}C NMR (D_2O, 75 MHz): δ 103.8, 103.65 (C-1^I, 1^V), 103.4 (3C) (C-1^{II}, 1^{III}, 1^{IV}), 85.4 (C-3^I), 85.2 (C-3^{II}), 85.0 (2C) (C-3^{III}, 3^{IV}), 76.8 (C-5^V), 76.45, 76.4 (5C) (C-3^V, 5^I, 5^{II}, 5^{III}, 5^{IV}), 74.3 (C-2^V), 74.1 (3C) (C-2^{II}, 2^{III}, 2^{IV}), 73.6 (C-2^I), 70.4 (C-4^V), 69.0, 68.9 (4C) (C-4^I, 4^{II}, 4^{III}, 4^{IV}), 61.55 (5C) (C-6^I, 6^{II}, 6^{III}, 6^{IV}, 6^V), 50.4 (–$CH_2CH(O)CH_2$), 44.2,

44.1 (–$CH_2CH(O)CH_2$); ESIMS: *m/z* 907 $[M+Na]^+$; Anal. Calcd for $C_{33}H_{56}O_{27}$: C, 44.80; H, 6.33. Found: C, 44.67; H, 6.40. Compound **3**: $[\alpha]_D$ +86.8 (*c* 1.1, $CHCl_3$); ^1H NMR (D_2O, 400 MHz): δ 5.23 (1H, d, $J_{1,2}$ 3.5 Hz, H-1′), 4.63–4.55 (5H, m, H-$1^{V, IV, III, II}$), 3.92–3.85 (5H, m), 3.75–3.33 (19H, m), 3.25–3.19 (6H, m), 3.20 (1H, m, –$CH_2CH_2CH(O)CH_2$, *S*), 3.06 (1H, m, –$CH_2CH_2CH(O)CH_2$, *R*), 2.56–2.81 (2H, m, –$CH_2CH_2CH(O)CH_2$); ^{13}C NMR (D_2O, 75 MHz): δ 103.8, 103.64 (C-1^I, 1^V), 103.0 (3C) (C-1^{II}, 1^{III}, 1^{IV}), 85.4 (C-3^I), 85.3 (C-3^{II}), 85.0 (2C) (C-3^{III}, 3^{IV}), 76.8 (C-5^V), 76.45, 76.35 (5C) (C-3^V, 5^I, 5^{II}, 5^{III}, 5^{IV}), 74.3 (C-2^V), 74.1 (3C) (C-2^{II}, 2^{III}, 2^{IV}), 73.1 (C-2^I), 70.6 (C-4^V), 69.0, 68.9 (4C) (C-4^I, 4^{II}, 4^{III}, 4^{IV}), 61.60 (5C) (C-6^I, 6^{II}, 6^{III}, 6^{IV}, 6^V), 50.0 (–$CH_2CH_2CH(O)CH_2$), 47.3, 47.0 (–$CH_2CH_2CH(O)CH_2$); ESIMS: *m/z* 921 $[M+Na]^+$; Anal. Calcd for $C_{34}H_{58}O_{27}$: C, 45.43; H, 6.46. Found: C, 45.60; H, 6.29.

4.3. Monocyte isolation

Human monocytes were obtained from 20 mL of fresh blood. The sample was diluted in an equal volume of sterile saline, layered over 20 mL of lymphocyte separating medium (Shanghai Reagent Co. Ltd., China) in a 50 mL polypropylene conical tube and centrifuged for 30 min at 1800 rpm at 25 °C. The mononuclear cells were aspirated from the liquid/liquid interface (5 mL), added to 10 mL of PBS-medium in a 15-mL polypropylene conical tube, and centrifuged for 10 min at 1200 rpm and 4 °C. The pellet was resuspended in 10 mL of PBS, centrifuged again for 10 min at 1200 rpm and 4 °C, and then resuspended in 20 mL of RMPI-1640 medium. The cell suspension was cultured in RMPI-1640 medium supplemented with 10% fetal calf serum, gentomycin, 100 U/mL penicillin/100 μg/mL streptomycin, and pyruvate at 37 °C in a 5% CO_2 atmosphere for 1 h. The monocytes were isolated with a cell scraper and diluted in 10 mL of RMPI-1640 medium. A small volume (10 μL) was diluted 10-fold into trypan blue for the counting and viability estimation with a hemocytometer. The cell suspension was then diluted in RMPI-medium to achieve the desired final cell density.

4.4. Animal studies

Twenty-four of C57BL/6 mice (18–22 g/each) were purchased from Hubei Province Laboratory Animal Center (China). The experimental animals were randomly divided into three groups (four males and four females for each group) and allowed 1 week to adapt to their environment before the experiments.

Samples of **1**, **2**, and **3** were dissolved in normal saline and sterilized again. A solution of each drug was administrated in one concentration by gavage stomach tube once daily. Gavaging volume was 0.2 mL for each mouse. Normal saline was used as control and 200 μg/mL of the dosage was used for every group. All mice were euthanized after a 14-day treatment. Phagocytosis by peritoneal macrophages was detected. Simultaneously, spleen lymphocytes were prepared at once for testing lymphocyte proliferation as described later.

4.5. Superoxide anion production activity

The measurement of the superoxide anions released from monocytes was based on the reduction of ferricytochrome *c* as assayed by the increase in its absorbencies at 550 nm.[19] The monocytes were cultured in RMPI-1640 medium supplemented with 10% fetal calf serum, gentomycin, penicillin/streptomycin, and pyruvate at 37 °C in a 5% CO_2 atmosphere. Monocytes (1×10^5 cells/well, 96-well polystyrene microtiter plate) were activated by **1**, **2**, or **3** (20 μg/mL) for 2 h at 37 °C, respectively. After the addition of ferricytochrome *c* (6×10^5 mol/L) to each well, the absorbencies values of individual wells were read at 550 nm with a microplate reader. In the test, a superoxide dismutase (100 μg/mL) was used as a blind well. Each value for optical density was calculated by subtracting the value of the control experiment without **1**, **2**, or **3** treatment. The nanomoles of $O_2^{\cdot -}$ production were then calculated as described in the literature.[19]

4.6. Phagocytosis by peritoneal macrophages

Phagocytosis by peritoneal macrophages was detected using the method described by Lin and Feng[20] with a small modification. Briefly, on the last day, 0.5 mL of 5×10^6 chicken red blood cells (CRBC) was intraperitoneally injected into each mouse, and the mice were euthanized 1 h later. The fluid from the abdominal cavity was collected to make a smear for each mouse. The smears were incubated at 37 °C for 30 min in a wet box, fixed with 95% ethanol, and then stained by Giemsa dye. The number of macrophages ingesting CRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytic rate (PR) and phagocytic index (PI) were calculated using the following formula:

$$\text{PR}\ (\%) = (\text{number of macrophages ingesting CRBC} / \text{number of total macrophages}) \times 100$$

$$\text{PI} = \text{number of total ingested CRBC} / \text{number of macrophages ingesting CRBC}$$

4.7. Preparation of spleen cell suspension and lymphocyte proliferation assay

Spleens were aseptically obtained from the mice and placed in complete RPMI 1640 medium. Then, the spleens were teased and filtered through steel mesh. A single-cell suspension was prepared. The cells were washed twice and then resuspended in complete RPMI 1640 medium. The concentration of splenocytes was adjusted to 5×10^6 cells/mL.

The proliferation ability of the lymphocytes was determined by MTT (3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide, Sigma) stain assay.[17] The 100 μL aliquots of lymphocytes were seeded into a 96-well polystyrene microtiter plate in the presence of mitogen Con

A (10 μg/mL). Three wells were used for each mouse. The plate was incubated for 48 h at 37 °C in a humidified 5% CO_2–air mixture. After addition of 10 μL of MTT per well, the plate was incubated for 4 h and then 100 μL of 20% sodium dodecyl sulfate (Sigma, SDS) was pipetted into each well to dissolve the formazan crystals. After incubation at 37 °C in a 5% CO_2 atmosphere overnight, the microplate was read on a microplate reader using the test wavelength of 570 nm.

4.8. Scavenging ability toward superoxide anion

Superoxide anions were generated by using a CL system of Me_2SO–NaOH–O_2–luminol.[18] The reaction mixture contained 100 mL NaOH (0.1 mol/L), 100 mL luminol (1×10^{-2} mol/L), and 700 mL phosphate buffer (pH = 8.6). Compound **1**, **2**, or **3** (100 mL) (0 μg/mL, 0.1 μg/mL) was injected into the mixture. The final volume was always the same (1 mL) for all assays. The sample cells were immediately placed in the Ultra-Weak Luminescence Analyzer (Beijing, China). The CL intensity was simultaneously recorded every 2 s by the processor. The sample without **1**, **2**, or **3** was also recorded for a control comparison.

4.9. Statistical analysis

All values were expressed as mean ± SD. Statistical analysis was performed by Student's *t*-test. A value of $p < 0.05$ was accepted as statistically significant.

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