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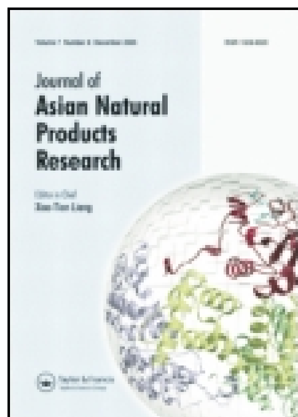


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# Microbial deglycosylation and ketonization of ginsenosides $Rg_1$ and $Rb_1$ by *Fusarium oxysporum*

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Two major ginsenosides, ginsenoside-$Rg_1$ (**1**) and ginsenoside-$Rb_1$ (**2**), were transformed by the fungus *Fusarium oxysporum* f. sp. *Lycopersici* (Z-001). **1** was converted into five metabolites, ginsenoside-$F_1$ (**3**), 6α,12β-dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**4**), 3a-oxa-3a-homo-6α,12β-dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**5**), 20(*S*)-protopanaxatriol (**6**), and 3-oxo-20(*S*)-protopanaxatriol (**7**). **2** was converted into four metabolites, ginsenoside-Rd (**8**), ginsenoside-$F_2$ (**9**), compound K (**10**), and 12β-hydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**11**). The structures of these metabolites were determined by the analysis of extensive spectroscopic data. Among them, **4** and **5** were two new compounds. Deglycosylation and ketonization at C-3 were recognized as the characteristic reactions of this strain.

**Keywords:** ginsenosides; *Fusarium oxysporum*; deglycosylation; ketonization

## 1. Introduction

Ginsenosides are the main effective constituents of *Panax* species, and they exhibit extensive pharmacological activities, including memory-enhancing, anticancer, immunity, and tumor multi-drug resistant reversal activities [1–4]. Ginsenoside-$Rg_1$ (**1**) is one of the major protopanaxatriol-type (PPT) saponins, and ginsenoside-$Rb_1$ (**2**) is one of the major protopanaxadiol-type saponins [5]. Several metabolic studies have shown that they could be transformed to more potent bioactive compounds such as PPT and compound K [6] by human intestinal microflora after oral administration [7,8]. Pharmacological experiments *in vitro* and *in vivo* suggested that the minor ginsenosides ($F_1$, $Rh_1$, $Rh_2$, compound K, etc.) and aglycons are more readily absorbed into the bloodstream and are pharmaceutically active [1,2,9]. The minor ginsenosides and aglycons can be produced from the major ginsenosides via partial or complete hydrolysis of the sugar moieties using traditional methods, such as heating, acid hydrolysis, and Smith degradation [10]. As we know, many varieties of microorganisms possess glycosidases, and microbial transformation has many advantages such as low cost, high yield, mild condition, and little pollution. The aims of this work were to identify a microorganism that can extensively and completely metabolize ginsenoside-$Rg_1$ (**1**) and ginsenoside-$Rb_1$ (**2**) and to chemically identify the metabolites. Meanwhile, we summarized the rules of the microbial conversion.

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## 2. Results and discussion

In the present study, 21 microbial strains were screened for the ability to convert ginsenoside-$Rg_1$ (**1**) and ginsenoside-$Rb_1$ (**2**), and one fungal strain, *Fusarium oxysporum* f. sp. *Lycopersici* Z-001, was found to be able to convert both compounds **1** and **2** extensively and completely (Table 1).

Ginsenoside-$Rg_1$ (**1**) was converted into three known metabolites and two new metabolites, and a plausible bioconversion pathway was proposed (Figure 1). The three known metabolites were identified as ginsenoside-$F_1$ (G-$F_1$, **3**) [11], 20(*S*)-protopanaxatriol (**6**) [12], and 3-oxo-20(*S*)-protopanaxatriol (**7**) [13] by spectroscopic data.

Metabolite **4**, a white amorphous powder, is less polar than **3** (G-$F_1$) by TLC analysis. HR-ESI-MS showed an $[M + Na]^+$ ion peak at *m/z* 659.4137, indicating the molecular formula of $C_{36}H_{60}O_9$, which is two hydrogen atoms fewer than **3** ($C_{36}H_{62}O_9$). The IR spectrum of **4** showed an absorption band at $1697\,cm^{-1}$, which indicated the presence of a carbonyl functional group. On the basis of the above MS and IR spectral data, it was deduced that an OH group was oxidized into a C=O group. In comparison with the $^1$H NMR spectrum of **3** [11], a distinctive signal of H-3 at $\delta_H$ 3.51 (m) disappeared, and the $^{13}$C NMR and DEPT spectra of **4** exhibited the appearance of one new carbon at $\delta_C$ 218.5 and the loss of an oxygen-bearing carbon ($\delta_C$ 78.5, C-3). These evidences indicated that **4** was an alcohol ketonized derivative of **3** at C-3. This was confirmed by the HMBC experiment, which showed correlations of two methyl protons of H-28 ($\delta_H$ 1.65), H-29 ($\delta_H$ 1.67), and H-2 ($\delta_H$ 2.27, 2.78) with the same carbonyl carbon ($\delta_C$ 218.5). On the basis of the above analysis, **4**

Table 1. Preliminary screening test of 21 microorganism strains.

| Microorganism | Medium[a] | $Rg_1$[b] | $Rb_1$[b] |
|---|---|---|---|
| *Aspergillus niger* Van Tieghem AS 3.739 | 1 | ++ | + |
| *Aspergillus niger* Van Tieghem AS 3.795 | 1 | ++ | − |
| *Aspergillus niger* Van Tieghem ZW-3 | 1 | ++ | − |
| *Aspergillus niger* Van Tieghem AS 3.1858 | 1 | − | − |
| *Absidia coerulea* AS 3.2462 | 1 | ++ | − |
| *Absidia coerulea* Bainier AS 3.3389 | 1 | ++ | − |
| *Absidia coerulea* Bainier AS 3.3538 | 1 | + | + |
| *Absidia coerulea* Bainier ZW-1 | 1 | − | − |
| *Absidia coerulea* Bainier ZW-2 | 1 | + | − |
| *Fusarium oxysporum* AS 3.3633 | 1 | − | + |
| *Fusarium sambucinum* AS 3.4602 | 1 | ++ | + |
| *Fusarium solani* (Martius) Saccardo AS 3.1829 | 1 | + | ++ |
| *Fusarium oxysporum* f. sp. *lycopersici* Z-001 | 1 | ++ | ++ |
| *Fusarium vasinfectum* Z-006 | 1 | − | + |
| *Fusarium oxysporum* f. sp. *cucumerinum* Z-007 | 1 | + | + |
| *Rhizopus stolonifer* AS 3.3463 | 1 | − | − |
| *Rhizopus stolonifer* (Ehrenberg ex Fries) Lind AS 3.2050 | 1 | − | − |
| *Streptomyces* sp. IAA-601 | 2 | − | − |
| *Streptomyces* sp. IAA-602 | 2 | − | + |
| *Streptomyces* sp. IAA-603 | 2 | ++ | − |
| *Enterobacter* sp. IMM-21 | 3 | − | + |

Notes: [a]Medium 1: potato (200 g), glucose (20 g), $KH_2PO_4$ (3.0 g), $MgSO_4$ (0.75 g), $VB_1$ micro amounts, water (1 l), pH 6.0. Medium 2: yeast extract (2 g), malt extract (10 g), glucose (4 g), water (1 l), pH 7.3. Medium 3: wheat bran (50 g), $K_2HPO_4$ (2 g), $(NH_4)_2SO_4$ (4 g), water (1 l), pH 6.0.
[b]'++' denotes many metabolites detected, '+' denotes few metabolites and '−' denotes no products yielded.

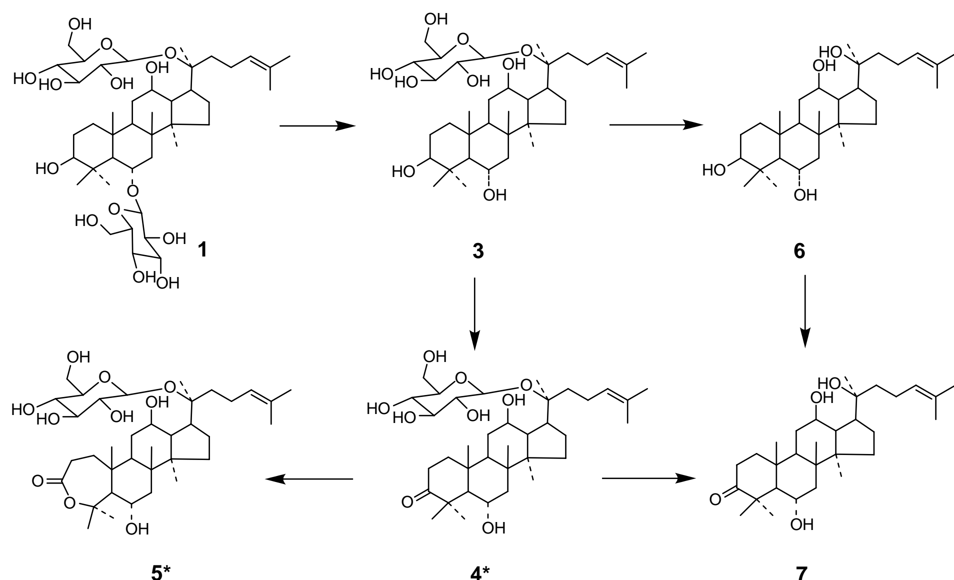


Figure 1. A proposed biotransformation pathway of ginsenoside $Rg_1$ (**1**) by *F. oxysporum* (*, new compound).

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was elucidated as 6α,12β-dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside.

The molecular formula of **5** was determined to be $C_{36}H_{60}O_{10}$ by HR-ESI-MS (*m/z* 675.4094 $[M + Na]^+$). The molecular weight of **5** was 16 amu more than that of **4**, indicating that an oxygen atom was introduced into the molecule. Compared with the IR absorption band at 1697 $cm^{-1}$ of carbonyl group in **4**, the absorption band at 1708 $cm^{-1}$ and the additional C—O—C absorption band at 1112 $cm^{-1}$ in **5** showed a tiny difference between the two compounds, which suggested the presence of an ester group in **5**. Compared with the $^{13}C$ NMR spectrum of **4**, the absence of carbonyl signal corresponding to C-3 at $\delta_C$ 218.5 was observed, whereas the presence of one lactone carbon at $\delta_C$ 174.0 as well as the downfield shift of C-4 at $\delta_C$ 86.3 indicated a lactone moiety in the A ring. It is most likely to be biogenetically derived by Baeyer–Villiger-type oxidation of a 3-keto ginsenoside derivative, such as **4**. Compared with the $^{13}C$ NMR spectral data of the similar structures, the chemical shifts $\delta_C$ 174.0 (C-3) and $\delta_C$ 86.3 (C-4) were in good accordance with those reported previously [14–16]. Thus, the structure of **5** was elucidated as 3a-oxa-3a-homo-6α,12β-dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside.

When we investigated the transformation of protopanaxatriol-type ginsenoside-$Rg_1$ (**1**) by *F. oxysporum*, it was founded that deglycosylation was the main reaction type. In addition, ketonization of OH group at C-3 was remarkable in compounds **4**, **5**, and **7**. To confirm that ketonization at C-3 is the characteristic reaction of this strain, protopanaxadiol-type ginsenoside-$Rb_1$ (**2**) was taken as substrate for the biotransformation with the same strain. As a result, four known metabolites were obtained, and they were identified as ginsenoside-Rd (**8**) [17], ginsenoside-$F_2$ (**9**) [18], compound K (**10**) [18], and 12β-hydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**11**) [19] on the basis of ESI-MS, $^1H$, and $^{13}C$ NMR spectral data. As expected, **11** was also a ketonized derivative at C-3. And, a plausible bioconversion pathway was also proposed (Figure 2). Thus, it might be concluded that this high regio-selective ketonization at C-3 was an additional characteristic reaction of

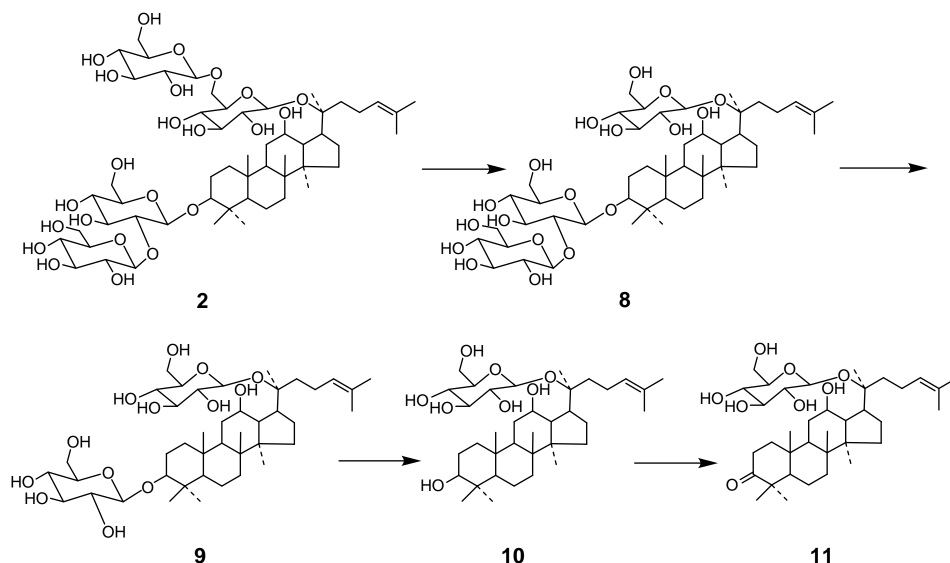


Figure 2. A proposed biotransformation pathway of ginsenoside $Rb_1$ (**2**) by *F. oxysporum*.

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this strain except for deglycosylation, when converting ginsenosides.

## 3. Experimental

### *3.1 General experimental procedures*

IR spectra were measured on Nicolet 5700 FT-IR spectrometer and Nicolet iN10 MX FT-IR microscope spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The $^{1}H$ and $^{13}C$ NMR spectra were recorded by Varian-400 and Bruker ARX-500 spectrometers in pyridine-$d_5$. Chemical shifts ($\delta$) are given in ppm, and coupling constants ($J$) are given in hertz (Hz). ESI-MS and HR-ESI-MS were obtained by a Q-trap ESI mass spectrometer (Applied Biosystems/MDS Sciex, Carlsbad, CA, USA). Column chromatography was performed with Amberlite XAD-16 macroporous resin (Rohm & Haas Co., Midland, MI, USA). Analytical HPLC was performed on an Agilent 1200 HPLC connected to a diode array detector, using an Apollo $C_{18}$ column (250 mm × 4.6 mm i.d., 5 μm). Semi-preparative reversed-phase HPLC was performed on a Shimadzu LC-6AD instrument with a Grace $C_{18}$ column (250 mm × 10 mm i.d., 5 μm) and a Shimadzu RID-10A detector. Analytical TLC was performed on pre-coated silica gel GF-254 plates (Qingdao Marine Chemical Co., Qingdao, China); developing solvent is chloroform–methanol–water (65:35:10, v/v/v, lower phase); and the visualization of TLC plates was done by spraying with 5% $H_2SO_4$ in EtOH followed by heating at 120°C. All solvents were of analytical reagent grade (Beijing Chemical Works, Beijing, China).

### *3.2 Substrates and organisms*

The substrate ginsenoside-$Rg_1$ and ginsenoside-$Rb_1$ were purchased from Shanghai Tauto Biotech (Shanghai, China) and the purity was found to be above 98% by HPLC analysis. The standard samples (ginsenosides $Rg_1$, $Rb_1$) were purchased from Shanghai Tauto Biotech and National Institutes for Food and Drug Control (Beijing, China), and the purity was above 98%. The strains (Table 1) numbered 'AS' were purchased from the Institute of Microbiology, Chinese Academy of Sciences; the others were our laboratory's own preservation. The culture media for screening and preparative biotransformation are also shown in Table 1.

### 3.3 Preliminary screening test

Preliminary screening was performed in 250 ml Erlenmeyer flasks containing 60 ml of medium. The flasks were placed on rotary shakers operating at 120 rpm at 26 ± 1°C. A standard two-stage fermentation protocol was employed in all experiments and the substrate was added to the incubation media 48 h after the inoculation of stage II in a 20 mg/ml *N*,*N*-dimethylformamide (DMF) solution at the final concentration of 60 mg/l in the medium. Culture controls consisted of fermentation blanks in which the organisms were grown under identical conditions but without adding the substrate. After 7 days of incubation, the cultures were filtered under reduced pressure, and the filtrate was subjected to column chromatography on

Table 2. $^1$H NMR and $^{13}$C NMR spectroscopic data for metabolites **4** and **5** ($\delta$ in ppm, *J* in Hertz).

| | Metabolite **4** | | Metabolite **5** | |
|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ |
| 1 | 39.8, $CH_2$ | 1.50 (m), 1.75 (m) | 33.2, $CH_2$ | 1.75 (m), 2.02 (m) |
| 2 | 33.3, $CH_2$ | 2.27 (m), 2.78 (m) | 32.3, $CH_2$ | 2.36[a], 2.80 (dt, 5.8, 14.0) |
| 3 | 218.5, C | | 174.0, C | |
| 4 | 47.6, C | | 86.3, C | |
| 5 | 58.9, CH | 1.88 (d, 10) | 57.4, CH | 1.87 (brs) |
| 6 | 66.7, CH | 4.21 (m) | 66.4, CH | 4.21 (m) |
| 7 | 45.4, $CH_2$ | 1.83 (m)[a] | 44.9, $CH_2$ | 1.83 (m)[a] |
| 8 | 40.5, C | | 40.2, C | |
| 9 | 48.6, CH | 1.60 (m)[a] | 51.1, CH | 1.62 (m)[a] |
| 10 | 38.1, C | | 39.9, C | |
| 11 | 31.6, $CH_2$ | 1.64[a], 2.00[a] | 33.1, $CH_2$ | 1.64[a], 2.04[a] |
| 12 | 69.9, CH | 4.16 (m) | 69.9, CH | 4.15 (t, 9.3) |
| 13 | 49.2, CH | 1.97[a] | 49.3, CH | 1.97[a] |
| 14 | 51.3, C | | 51.2, C | |
| 15 | 30.7, $CH_2$ | 1.03[a], 1.60[a] | 30.6, $CH_2$ | 1.00[a], 1.60[a] |
| 16 | 26.5, $CH_2$ | 1.37 (m), 1.83[a] | 26.4, $CH_2$ | 1.37 (m), 1.83[a] |
| 17 | 51.3, CH | 2.63 (m) | 51.2, CH | 2.61 (m) |
| 18 | 16.2, $CH_3$ | 1.00 (s) | 15.8, $CH_3$ | 1.19 (s) |
| 19 | 17.7, $CH_3$ | 0.81 (s) | 19.9, $CH_3$ | 0.96 (s) |
| 20 | 83.2, C | | 83.2, C | |
| 21 | 22.2, $CH_3$ | 1.61 (s) | 22.3, $CH_3$ | 1.61 (s) |
| 22 | 36.1, $CH_2$ | 1.80[a], 2.39 (m) | 36.2, $CH_2$ | 1.80[a], 2.45 (m) |
| 23 | 23.1, $CH_2$ | 2.22[a], 2.50 (m) | 23.1, $CH_2$ | 2.22 (m), 2.51[a] |
| 24 | 125.9, CH | 5.23 (t, 6.8) | 125.9, CH | 5.24 (t, 6.6) |
| 25 | 130.9, C | | 130.9, C | |
| 26 | 25.7, $CH_3$ | 1.58 (s) | 25.7, $CH_3$ | 1.58 (s) |
| 27 | 17.7, $CH_3$ | 1.58 (s) | 17.7, $CH_3$ | 1.57 (s) |
| 28 | 32.0, $CH_3$ | 1.65 (s) | 33.1, $CH_3$ | 1.82 (s) |
| 29 | 19.9, $CH_3$ | 1.67 (s) | 27.4, $CH_3$ | 1.91 (s) |
| 30 | 17.2, $CH_3$ | 0.97 (s) | 17.1, $CH_3$ | 1.03 (s) |
| 1′ | 98.2, CH | 5.18 (d, 7.6) | 98.2, CH | 5.18 (d, 8.0) |
| 2′ | 75.1, CH | 3.99 (dt, 3.6, 7.6) | 75.0, CH | 4.00 (t, 8.0) |
| 3′ | 79.4, CH | 4.24 (m) | 79.4, CH | 4.23 (m) |
| 4′ | 71.7, CH | 4.16 (m) | 71.7, CH | 4.15 (m) |
| 5′ | 78.3, CH | 3.92 (m) | 78.3, CH | 3.92 (m) |
| 6′ | 62.9, $CH_2$ | 4.31 (m), 4.47 (m) | 62.9, $CH_2$ | 4.32 (dd, 5.4, 11.6), 4.49 (dd, 2.3, 11.6) |

Note: [a] Overlapping signals.

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Amberlite XAD-16 macroporous resin (1.0 g), eluting with $H_2O$ and 80% ethanol successively. The 80% ethanol fraction was concentrated under reduced pressure to dryness, and the obtained residue was dissolved in methanol and then analyzed by TLC.

### 3.4 Preparative scale biotransformation

The 2-day-old Z-001 seed culture was added to 1000 ml Erlenmeyer flasks containing 300 ml of PDA medium according to the standard two-stage fermentation protocol. After 2 days incubation, 10 l of the cultures was centrifuged (8000*g*, 10 min) to obtain the mycelia as crude enzymes. Then, the mycelia were re-suspended in the phosphate buffer (0.05 M, pH 6.0) in 500 ml Erlenmeyer flasks. Ginsenoside-$Rg_1$ (**1**) (400 mg) in 4 ml DMF was uniformly added into eight flasks at a final concentration of 300 mg/l. After 7 days of incubation, the reaction broth was pooled and centrifuged. The supernatant was subjected to column chromatography on Amberlite XAD-16 macroporous resin (120 g), eluting with $H_2O$, 20% ethanol, 50% ethanol, 80% ethanol and 100% ethanol, successively. The residue of 80% ethanol fraction was concluded to contain the main substrate and metabolites by TLC analysis. Then this fraction (220.0 mg) was further purified by semi-prep. RP-HPLC, yielding substrate ginsenoside-$Rg_1$ (**1**) (33.3 mg, 55% methanol, $t_R$ 15.2 min), metabolites **3** (21.4 mg, 65% methanol, $t_R$ 22.8 min), **4** (78.3 mg, 65% methanol, $t_R$ 26.2 min), **5** (4.0 mg, 38% acetonitrile, $t_R$ 8 min), **6** (2.0 mg, 75% methanol, $t_R$ 23 min), and **7** (7.7 mg, 75% methanol, $t_R$ 25.5 min).

The procedure for transformation of ginsenoside-Rb1 and isolation of the metabolites was performed as described above. Finally, metabolites **8** (14.0 mg, 35% acetonitrile, $t_R$ 10.7 min), **9** (130.7 mg, 45% acetonitrile, $t_R$ 9 min), **10** (37.3 mg, 50% acetonitrile, $t_R$ 22 min), and **11** (3.4 mg, 58% acetonitrile, $t_R$ 16 min) were obtained, and no substrate recovered.

#### *3.4.1 6α,12β-Dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**4**)*

White amorphous powder; IR (KBr) $\nu_{max}$: 3392, 2960, 1697, 1381, 1039 $cm^{-1}$; for $^1H$ NMR (pyridine-$d_5$, 400 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 100 MHz) spectroscopic data see Table 2; HR-ESI-MS *m/z* 659.4137 $[M + Na]^+$ (calcd. for $C_{36}H_{60}O_9Na$, 659.4130).

#### *3.4.2 3a-Oxa-3a-homo-6α,12β-dihydroxydammar-3-one-20(*S*)-*O*-β-D-glucopyranoside (**5**)*

White amorphous powder; IR $\nu_{max}$: 3364, 2964, 1708, 1385, 1112, 1043 $cm^{-1}$; for $^1H$ NMR (pyridine-$d_5$, 500 MHz) and $^{13}C$ NMR (pyridine-$d_5$, 125 MHz) spectroscopic data see Table 2; HR-ESI-MS *m/z* 675.4094 $[M + Na]^+$ (calcd. for $C_{36}H_{60}O_{10}Na$, 675.4084).


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