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Biotransformation of Ginsenoside Rb1 to Ginsenoside Rd and 7 Rare Ginsenosides Using *Irpex lacteus* with HPLC-HRMS/MS Identification

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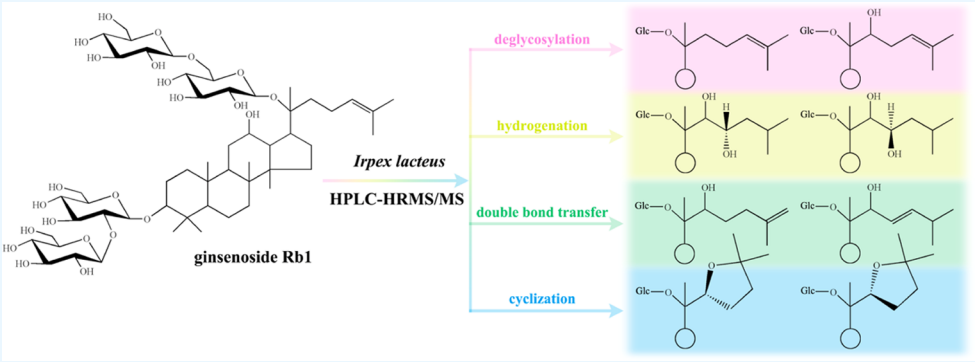

ABSTRACT: The biotransformation of ginsenosides using microorganisms represents a promising and ecofriendly approach for the production of rare ginsenosides. The present study reports on the biotransformation of ginsenoside Rb1 using the fungus *Irpex lacteus*, resulting in the production of ginsenoside Rd and seven rare ginsenosides with novel structures. Employing high-performance liquid chromatography coupled with high-resolution tandem mass spectrometry, the identities of the transformation products were rapidly determined. Two sets of isomers with molecular weights of 980.56 and 962.55 were discovered among the seven rare ginsenosides, which were generated through the isomerization of the olefin chain in the protopanaxadiol (PPD)-type ginsenoside skeleton. Each isomer exhibited characteristic fragment ions and neutral loss patterns in their tandem mass spectra, providing evidence of their unique structures. Time-course experiments demonstrated that the transformation reaction reached equilibrium after 14 days, with Rb1 initially generating Rd and compound **5**, followed by the formation of other rare ginsenosides. The biotransformation process catalyzed by *I. lacteus* was found to involve not only the typical deglycosylation reaction at the C-20 position but also hydroxylation at the C-22 and C-23 positions, as well as hydrogenation, transfer, and cyclization of the double bond at the C-24(25) position. These enzymatic capabilities extend to the structural modification of other PPD-type ginsenosides such as Rc and Rd, revealing the potential of *I. lacteus* for the production of a wider range of rare ginsenosides. The transformation activities observed in *I. lacteus* are unprecedented among fungal biotransformations of ginsenosides. This study highlights the application of a medicinal fungi-based biotransformation strategy for the generation of rare ginsenosides with enhanced structural diversity, thereby expanding the variety of bioactive compounds derived from ginseng.

1. INTRODUCTION

Ginseng is the dried root or rhizome of *Panax ginseng* C. A. Mayer (Araliaceae). It has been widely used for thousands of years in East Asia to treat a wide range of ailments and is gradually being used as a food and nutraceutical all over the world.[1−4] Ginsenosides are considered to be the main components responsible for the various biological activities of ginseng.[5−8] They have a wide variety of structures and can be classified as protopanaxadiol (PPD), protopanaxatriol (PPT), and oleanolic acid (OA) types depending on their sapogenins.[9] In naturally grown ginseng, four ginsenosides, including the PPT-type ginsenosides Re and Rg1, the PPD-type ginsenoside Rb1, and the OA-type ginsenoside Ro, accounted for more than 70% of the total ginsenoside content.[10] They are, therefore, known as the major ginsenosides. Accordingly, those present in trace or undetectable amounts in natural sources are generally defined as rare ginsenosides, which are mainly obtained by the transformation of major ginsenosides. Rare ginsenosides have fewer saccharide moieties or more functional groups attached to the sapogenins

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in terms of their structure, which results in better bioavailability and more pharmacological activity than the major ginsenosides.[11,12] For example, ginsenoside Rh2 is proven to have prominent matrix metalloproteinase inhibitory, anti-inflammatory, and antioxidant activities.[13] Ginsenoside Rh4 plays an anticancer role by inducing apoptosis and autophagic cell death.[14]

Transformation methods of ginsenosides have received increasing attention in the past decade. Many methods, including chemical transformation, enzymatic transformation, and microbial transformation, have been successively invented. Of these methods, chemical transformation has the advantages of high efficiency and low cost, but it is also limited by multiple byproducts and high environmental pollution.[15−19] Enzymatic transformation is advantageous in terms of short reaction time and high product purity, but the reaction conditions are difficult to control and the separation and purification of the enzyme are complicated.[20−24] Microbial transformation has the advantages of mild reaction conditions, strong substrate specificity, few byproducts, and environmental benign compared to other methods and is considered the most promising method for the preparation of rare ginsenosides.[25−27] Previous studies have shown that the isolated mutant filamentous fungus *Paecilomyces bainier* 229−7 was able to transform ginsenoside Rb1 to Rd with high selectivity and substrate tolerance.[28] A larger number of microorganisms such as *Aspergillus niger* XD101,[29] *Lactobacillus rossiae* DC05,[30] *Aspergillus tubingensis*,[31] and *Lactobacillus paralimentarius* LH4[32] are also discovered to have the ability to efficiently and stably transform ginsenoside Rb1 to the important pharmacologically active ginsenoside CK.

In addition to the preparation of known rare ginsenosides with good activities, such as CK and Rh2, the discovery of functional molecules with novel structures is also an important focus of ginsenoside biotransformation. The deglycosylation reaction has been the primary concern in the biotransformation pathway for a long time. However, only a limited variety of rare ginsenosides could be obtained by this reaction alone.[33] The olefin chain of the dammarane skeleton is susceptible to many reactions such as hydration, dehydration, isomerization, cyclization, and oxidation to generate a wide variety of sapogenins.[15,34,35] Combined with site-specific deglycosylation, skeleton modification can greatly increase the structural types of ginsenosides and provide a wealth of novel molecules to select for pharmacological studies.

Structural identification is the primary challenge to be addressed for the rare ginsenosides obtained after biotransformation. Solutions to this challenge usually rely on thin-layer chromatography, nuclear magnetic resonance, high-performance liquid chromatography (HPLC), capillary electrophoresis, and single-crystal X-ray diffraction.[36] One of these techniques that combines convenience and accuracy is HPLC coupled with high-resolution mass spectrometry (HRMS) and tandem mass spectrometry (MS/MS).[37,38] By determining precise molecular weights, HRMS allows for the derivation of both the possible elemental composition and the chemical formula of the ginsenosides, while MS/MS employs collision-induced dissociation (CID) to convert the kinetic energy of ions into internal energy, resulting in the dissociation of ginsenoside ions at active sites. This generates characteristic ion fragmentation and neutral loss. By integrating all of this information, it is possible to reveal the complete profile of ginsenoside molecules.[16] In addition, HPLC-MS provides structural information without the need for high purity and large amounts of sample, which plays a key role in the rapid structural identification of transformed ginsenosides.

In this study, the biotransformation of PPD-type ginsenosides Rb1 was carried out using the culture medium of the medicinal fungus *Irpex lacteus*. The biotransformation products were identified by HPLC combined with HRMS/MS, which revealed seven rare ginsenosides with novel structures in addition to the known ginsenoside Rd. The transformation pathway was investigated based on time-course experiments, revealing hydroxylation of the olefin chain, hydrogenation, transfer, and cyclization of the double bond, as well as conventional deglycosylation of the outer glycosyl substituent attached to C-20. *I. lacteus* was also extended to the biotransformation of ginsenosides Rc and Rd to verify its biotransformation activity. To the best of our knowledge, this transformation characteristic of *I. lacteus* is reported for the first time in fungi. The medicinal-fungus-based biotransformation strategy employed in this work may provide alternative pathways for the production of rare ginsenosides with novel structures.

2. RESULTS AND DISCUSSION

2.1. Structural Identification of the Biotransformation Products of Ginsenoside Rb1 by HPLC-HRMS/MS.

Ginsenoside Rb1 was biotransformed by incubation with the culture medium of *I. lacteus*. The transformation product was first investigated by HPLC-HRMS in full scan mode. As shown in the total ion chromatogram (TIC) in Figure 1, a total of

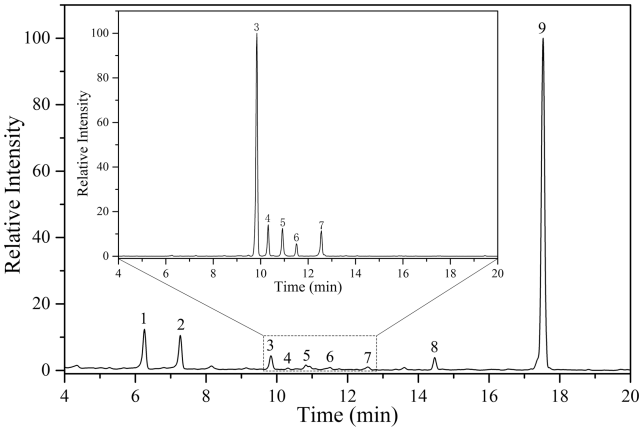

Figure 1. TIC of the biotransformation products of ginsenoside Rb1 generated by incubation with the culture medium of *I. lacteus*. The inset is the EIC of the ion at m/z 961.5392.

nine well-separated peaks are observed and defined as compounds **1**−**9** in order of retention time. By comparison of the retention time, molecular weight, and tandem mass spectra with the authentic standards, compound **8** is identified as the incompletely reacted ginsenoside Rb1. And the main product compound **9** is identified as ginsenoside Rd with a molecular weight of 946.55. It is formed by the deglycosylation of the outer glucose substituent attached to the C-20 position of Rb1. Besides the main product Rd, seven byproducts, compounds **1**−**7**, are also obtained. The peak areas of compounds **3**−**7** are so small as to be unnoticeable. However, in the extracted ion chromatogram (EIC) of their base peak ion at m/z 961.5392, five clearly separated peaks of symmetrical shape can be observed, as shown in the inset of

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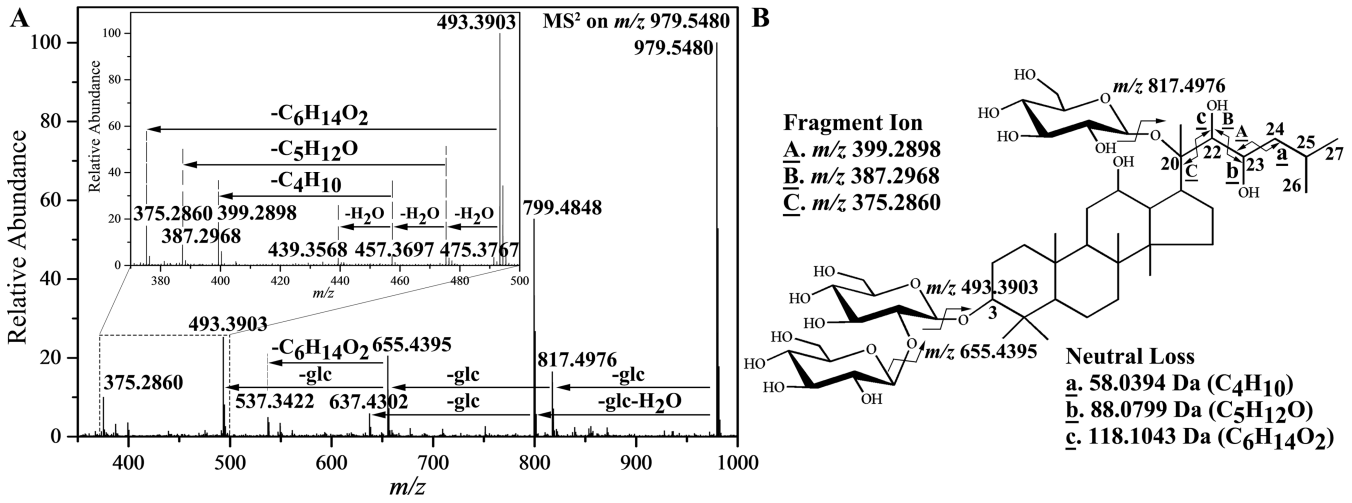

Figure 2. HRMS/MS spectrum from the $[M - H]^-$ ion at m/z 979.5480 (A) and the fragmentation pathway (B) of compound **1**.

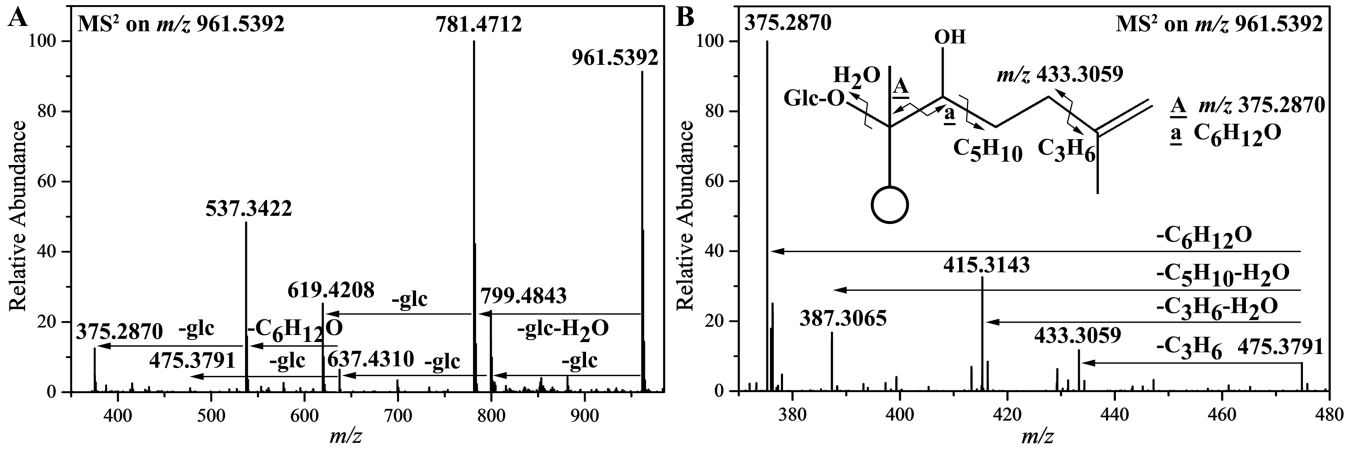

Figure 3. HRMS/MS spectrum from the $[M - H]^-$ ion at m/z 961.5392 (A) and magnified spectrum of the low m/z region and fragmentation pathway (B) of compound **3**.

Figure 1. These seven byproducts cannot match the available ginsenoside standards in terms of molecular weight and retention time. Therefore, HRMS/MS analysis is further used to collect their fragmentation information and thus infer their structures.

Compounds **1** and **2** are known to be isomers with molecular weights of 980.56 through the $[M + HCOO]^-$ ion at m/z 1025.5537 and the $[M - H]^-$ ion at m/z 979.5480, corresponding to a molecular formula of $C_{48}H_{84}O_{20}$. Their tandem mass spectra are identical, as shown in Figures 2A and S1. The tandem mass spectrum of the $[M - H]^-$ ion of compound **1** is analyzed as an example for structural identification. The neutral loss between these four adjacent ions, including the precursor ion at m/z 979.5480 and the product ions at m/z 817.4976, m/z 655.4395, and m/z 493.3903, is ca. 162.05 Da, indicating that the three product ions are formed by sequential dissociation of one glucose substituent from the precursor ion.[15] Compound **1** should therefore have a total of three glucose substituents, and the ion at m/z 493.3903 is its deprotonated aglycone ion. The product ions at m/z 799.4848 and m/z 637.4302 are the dehydration ions of m/z 817.4976 and m/z 655.4395, respectively. Their relative intensities indicate that compound **1** is more susceptible to the removal of one glucose substituent rather than two glucose substituents. This suggests that the more readily dissociable C-20 position of the sapogenin is attached to one glucose substituent, while the more stable C-3 position is attached to the other two glucose substituents.

The aglycone ion of compound **1** at m/z 493.3903 is 34.007 Da more than that of Rb1 at m/z 459.3833, suggesting the possible involvement of addition and oxidation reactions in the biotransformation process. As shown in Figure 2B, the product ion at m/z 375.2860 is one of the characteristic fragment ions of PPD-type ginsenosides, formed by the dissociation of the olefin chain at the C-20 position. Its presence suggests that the possible reactions all occurred on the olefin chain rather than on the tetracyclic skeleton of the sapogenin. In addition, the neutral loss between the ion at m/z 375.2860 and the aglycone ion at m/z 493.3903 Da is 118.1043 Da, corresponding to the molecular formula $C_6H_{14}O_2$, which indicates that the olefin chain of compound **1** is saturated and attached to two hydroxyl groups. The ions at m/z 475.3767, m/z 457.3697, and m/z 439.3568 are formed by the removal of one, two, and three H_2O molecules, respectively, from the aglycone ion. The neutral loss between the ions at m/z 457.3697 and m/z 399.2898 and between the ions at m/z 475.3767 and m/z 387.2968 is 58.0803 and 88.0799 Da, respectively, corresponding to the molecular formula of C_4H_{10} and $C_5H_{12}O$. The only possible structure for C_4H_{10} is the isobutane formed by the cleavage of the single bond between C-23 and C-24, while the

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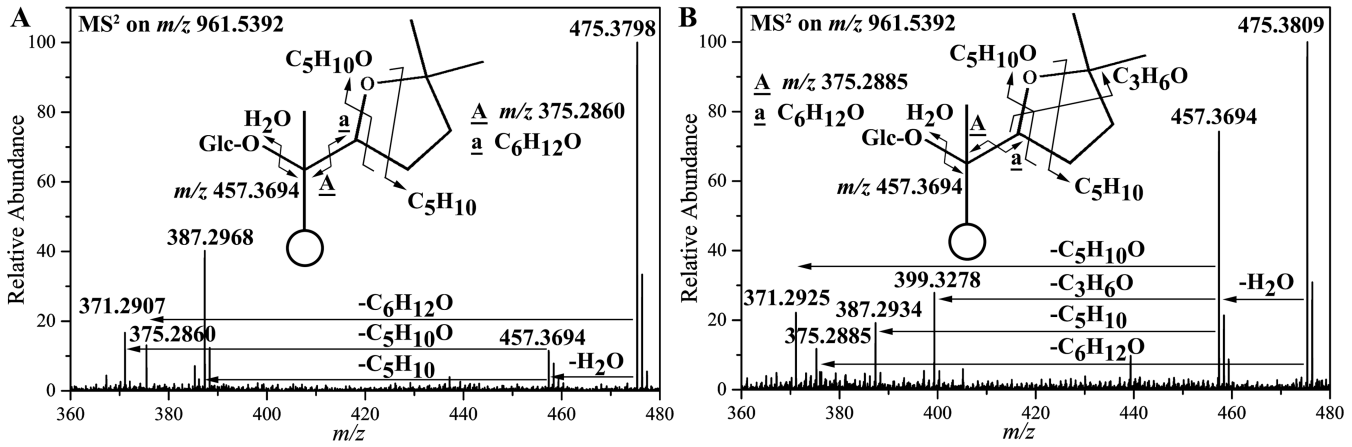

Figure 4. HRMS/MS spectra from the $[M\text{-}H]^-$ ion at m/z 961.5392 in the low m/z region and fragmentation pathways of compounds **4** (A) and **7** (B).

structure for $C_5H_{12}O$ can only be isopentanol formed by the cleavage of the single bond between C-22 and C-23, as depicted in Figure 2B. From the two neutral loss molecules, it could be determined that one hydroxyl group is attached at the C-23 position, leaving the other hydroxyl group only able to be attached at the C-22 position. Based on the analysis performed, it is concluded that compound **1** is derived from the biotransformation of Rb1 through deglycosylation of the outer glucose substituent at C-20, saturation of the C-24(25) double bond, and hydroxylation of C-22 and C-23. Accordingly, compound **1** is identified as (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-12, 22, 23(*S*/*R*)-trihydroxydammar-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside, and compound **2** is its C-23 epimer. Their structures are shown in Figure 2B.

Compounds **3**−**7** are five isomers with molecular weights of 962.55 and a molecular formula of $C_{48}H_{82}O_{19}$. The tandem mass spectra of the $[M - H]^-$ ion of compound **3** are shown in Figure 3. Figure 3A shows that the four ions at m/z 961.5392, m/z 799.4843, m/z 637.4310, and m/z 475.3791 all differ by ca. 162.05 Da from their adjacent ions. In addition, the relative intensities between the ions at m/z 799.4843 and m/z 637.4310 and between the dehydrated ions at m/z 781.4712 and m/z 619.4208 indicate that compound **3** consists of the same three glucose substituents as compound **1**, one of which is attached at the C-20 position and the other two at the C-3 position.

The ion at m/z 475.3791 is the deprotonated aglycone ion of compound **3**. As shown in Figure 3B, there is a neutral loss of 100.0921 Da between it and the characteristic fragment ion at m/z 375.2870, corresponding to the molecular formula $C_6H_{12}O$. This indicates that the tetracyclic skeleton of Rb1 is preserved in the transformation process, while the olefin chain is unsaturated and composed of one hydroxyl group. The neutral loss between the aglycon ion and the product ion at m/z 433.3059 is 42.0732 Da, equivalent to the molecular formula of C_3H_6, that is, the propylene resulting from the cleavage of the bond at C-24(25). The original bond at this site of PPD-type ginsenoside is a double bond of higher energy, which excludes the possibility of its cleavage in CID.[38] Therefore, it can be inferred that compound **3** underwent a double bond transfer reaction, where the double bond was transferred from the C-24(25) position to the C-25(26) position.

The neutral loss between the aglycon ion and the product ion at m/z 387.3065 is 88.0726 Da, which corresponds to the molecular formula of $C_5H_{12}O$. Due to the presence of the unsaturated double bond at the C-25(26) position, $C_5H_{12}O$ is considered to consist of one unsaturated C_5H_{10} molecule and one H_2O molecule, as is the case with the ion at m/z 415.3143, which is formed by the loss of one unsaturated C_3H_6 molecule and one H_2O molecule from the aglycone ion. The structure for C_5H_{10} can only be 2-methylbutene produced by the single bond cleavage at the C-22(23) position, suggesting that there is no hydroxyl group attached from the C-23 to C-27 positions in compound **3**. As a result, the only hydroxyl group on the olefin chain should be attached at the C-22 position. Based on the above analysis, compound **3** can be assigned to (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-12, 22-dihydroxydammar-25-en-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside derived from Rb1 through deglycosylation of the outer glucose substituent at C-20, transfer of the C-24(25) double bond, and hydroxylation of C-22.

As shown in Figure S2, compounds **4**−**7** have the same deglycosylated ions at m/z 961.5392, m/z 799.4843, m/z 637.4310, and m/z 475.3791 with similar relative intensities to compound **3**. This suggests that their glycosyl substituents are of the same number and position as in compound **3** and that the ion at m/z 475.3791 is their aglycone ion. Combined with the characteristic ion at m/z 375.2860 in the tandem spectrum of each compound, it can be inferred that the structural difference between these five isomers is only in the olefin chain and not in the tetracyclic skeleton. Moreover, the neutral loss between the ion at m/z 375.2860 and the aglycone ion at m/z 475.3791 corresponds to the $C_6H_{12}O$ molecule, indicating that each of the five isomers has an oxygen atom attached to the olefin chain. Notably, the product ion at m/z 537.3422 appears in all tandem mass spectra of compounds **1**−**7**. This fragment ion is formed by the removal of the $C_6H_{12}O$ molecule, namely, the olefin chain at the C-20 position, from the ion at m/z 637.4310 with the retention of one glucose substituent. Its appearance is not very common in the dissociation patterns of the major ginsenosides[16,37,38] but is a direct indication of the preservation of the tetracyclic skeleton and the presence of the oxidized olefin chain as a result of biotransformation.

The tandem spectrum of compound **4** in the range of m/z 360.0−480.0 is shown in Figure 4A. The ion at m/z 457.3694 is the dehydrated aglycone ion. It has a neutral loss of 70.0726 and 86.0787 Da with the ions at m/z 387.2968 and m/z 371.2907, respectively, corresponding to the molecular formula

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of C_5H_{10} and $C_5H_{10}O$. The neutral loss involving five carbon atoms is necessarily due to the cleavage of the C-22(23) single bond within the olefinic chain. If the olefin chain is linear, dissociation at the C-22(23) position cannot produce both an oxygenated fragment ($C_5H_{10}O$) and a nonoxygenated fragment (C_5H_{10}). Thus, the olefin chain of compound **4** is definitely not linear but cyclic with an oxygen atom attached to the C-22 position. It can therefore be concluded that compound **4** is the product of the cyclization reaction of the C-22 hydroxyl group with the C-24(25) double bond, that is, (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-22(*S*/*R*), 25-epoxydammarane-12-ol-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside. Its structural formula is shown in Figure 4A. The neutral loss of C_5H_{10} is produced by the simultaneous cleavage of the C-22(23) and C-25(O) bonds, while the neutral loss of $C_5H_{10}O$ is produced by the simultaneous cleavage of the C-22(23) and C-22(O) bonds. Moreover, if two neutral loss molecules produced from the aglycone ion differ by only one oxygen atom, a cyclic structure in the olefin chain would be expected.

Compound **7** has the same product ions as compound **4** except for an additional ion at *m*/*z* 399.3278, as shown in Figure 4B. The neutral loss between the additional ion and the dehydrated aglycone ion at *m*/*z* 457.3694 is 58.0416 Da, corresponding to the molecular formula of C_3H_6O. Its appearance provided further evidence for the cyclic structure of the olefin chain, as only a cyclic chain is capable of producing the neutral loss of C_3H_6O, $C_5H_{10}O$, and C_5H_{10} simultaneously. Compound **7** is therefore identified as the epimer of compound **4** derived from the chiral carbon atom at the C-22 position, namely, (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-22(*R*/*S*), 25-epoxydammarane-12-ol-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside. The ion at *m*/*z* 399.3278 is formed by cleavage of the C-22(23) and C-24(25) bonds, as shown in the structural formula in Figure 4B. Although the C-20 ginsenoside epimers of a sinister configuration eluted earlier than their rectus counterparts in the C18 column,[34,35] there are no reports on the relative retention times of the ginsenoside epimers derived from other chiral carbon atoms. The configurations of the chiral carbon atoms at C-22 in compounds **4** and **7** remain to be determined.

As shown in Figure 5, in addition to the dehydrated aglycone ion at *m*/*z* 457.3694 and the olefin chain dissociation

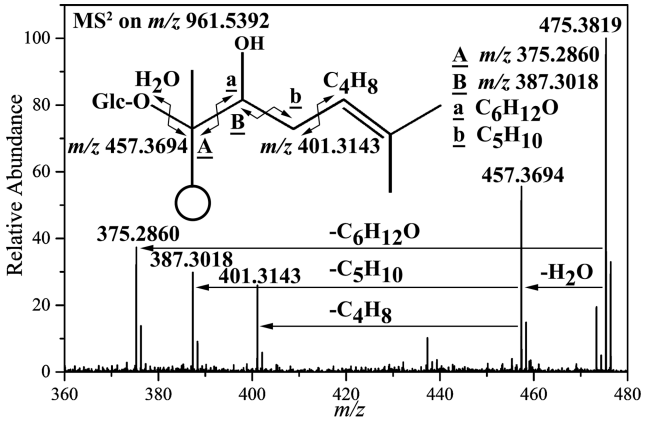

Figure 5. HRMS/MS spectrum from the [M-H]⁻ ion at *m*/*z* 961.5392 in the low *m*/*z* region and fragmentation pathway of compound **5**.

ion at *m*/*z* 375.2860, compound **5** predominantly generated two ions at *m*/*z* 401.3143 and *m*/*z* 387.3018 in the range of *m*/*z* 360.0−480.0. As with compound **3**, the ion at *m*/*z* 387.3018 is formed by the neutral loss of the C_5H_{10} molecule from the aglycone ion at *m*/*z* 475.3819. Its presence indicates that the oxygen atom is attached to form a hydroxyl group at the C-22 position. In addition, the neutral loss of 74.0676 Da between the ion at *m*/*z* 401.3143 and the aglycone ion corresponds to the C_4H_8 molecule, resulting from the single bond cleavage at C-23(24). This indicates that the double bond on the olefin chain remains at the C-24(25) position and is not involved in the double bond transfer or cyclization reaction. Therefore, compound **5** is identified as the deglycosylation and C-22 hydroxylation product of Rb1, that is, (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-12, 22-dihydroxydammar-24-en-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside with the structural formula shown in Figure 5. The single bond cleavage at C-23(24) with neutral loss of the C_4H_8 molecule is extremely rare in the CID of the major PPD-type ginsenosides. Its occurrence in compound **5** is possibly due to the inductive electron-withdrawing effect of both the hydroxyl group at the C-22 position and the double bond at the C-24(25) position, resulting in a weakened single bond at the C-23(24) position. On the other hand, the single bond at the C-23(24) position is preserved in compound **3**, with no neutral loss of the C_4H_8 molecule observed (Figure 3). This may be because the inductive effect of these two functional groups is attenuated along the carbon chain, and the single bond at the C-23(24) position is less affected and retains stability. This also indicates that the single bonds associated with electron-withdrawing functional groups are more easily dissociated in the CID.

The tandem mass spectrum of compound **6** in the range of *m*/*z* 360.0−480.0 is shown in Figure 6. Two new fragment

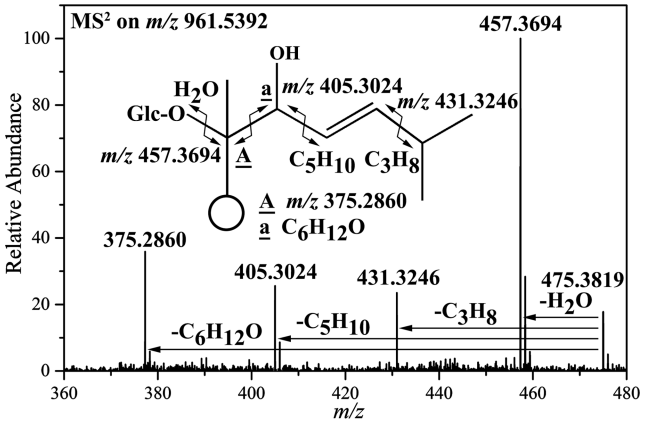

Figure 6. HRMS/MS spectrum from the [M − H]⁻ ion at *m*/*z* 961.5392 in the low *m*/*z* region and fragmentation pathway of compound **6**.

ions are observed at *m*/*z* 431.3246 and *m*/*z* 405.3024. The neutral loss between them and the aglycone ion at *m*/*z* 475.3819 is 44.0573 and 70.0795 Da, corresponding to the molecular formula of C_3H_8 and C_5H_{10}, respectively. The saturated C_3H_8 molecule could only be generated by bond cleavage at the C-24(25) position, suggesting that the C-24(25) bond is a single bond and that the original double bond is transferred but not to the C-25(26) and C-25(27) positions. The unsaturated C_5H_{10} molecule is generated by the single

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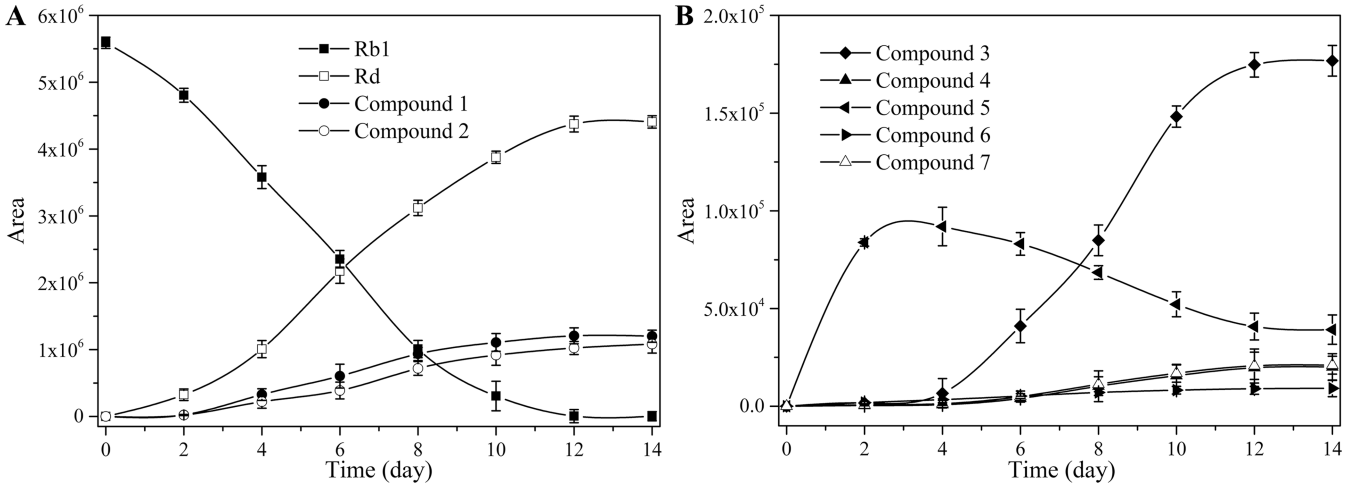
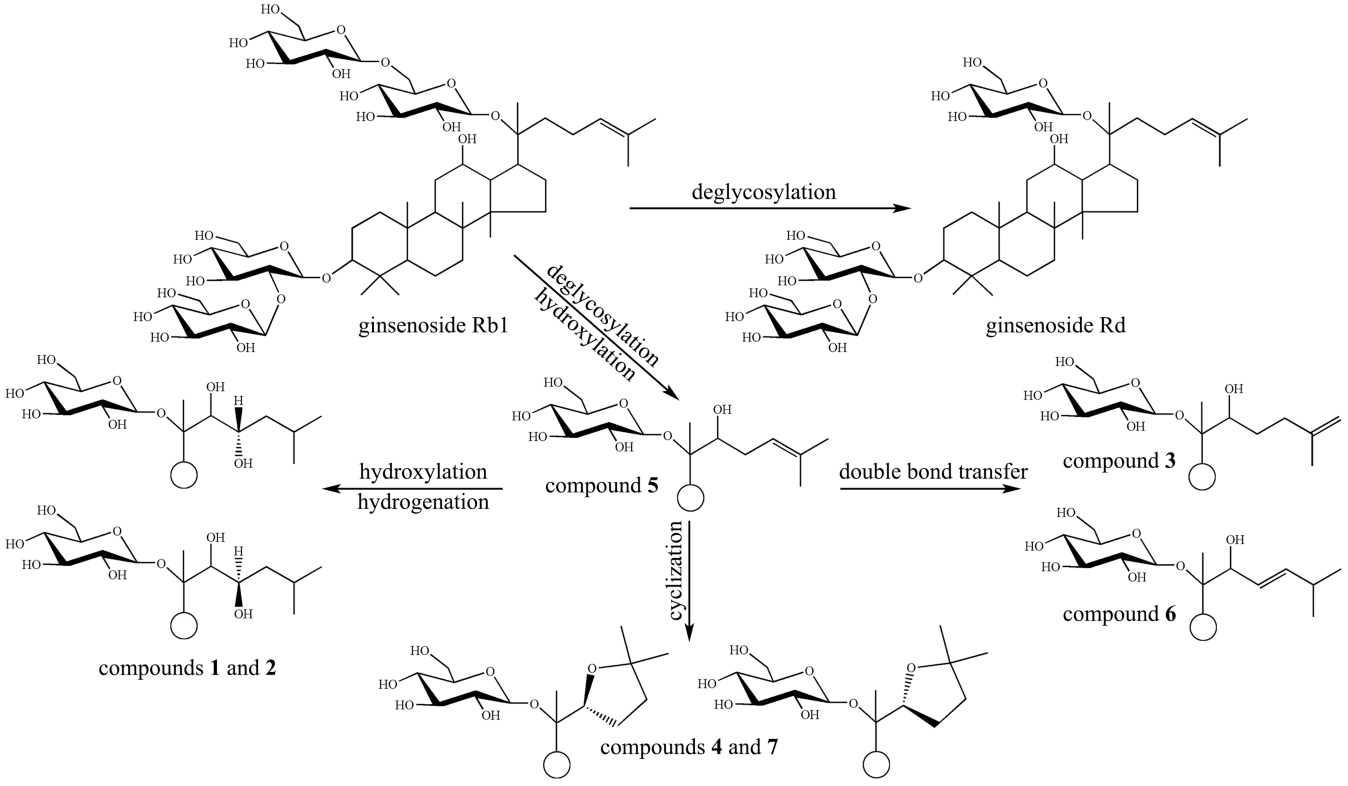


Figure 7. Time-course variation in ginsenoside Rb1 and its transformation products during the biotransformation process.

Scheme 1. Biotransformation Pathway of Ginsenoside Rb1 by Incubation with the Culture Medium of *I. lacteus*



bond cleavage at the C-22(23) position, which rules out the possibility of the double bond transfer to the C-22(23) position and also the possibility of the oxygen atom being attached from the C-23 to C-27 positions. Therefore, the double bond can only be located at the C-23(24) position, and the oxygen atom can only be attached at the C-22 position. From the above analysis, compound **6** can be identified as (3*β*, 12*β*)-20-(*β*-D-glucopyranosyloxy)-12, 22-dihydroxydammar-23-en-3-yl 2-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranoside with its structural formula depicted in Figure 6.

Information on all of the transformation products and the major ions in their mass spectra is summarized in Table S1. Overall, Rb1 was biotransformed to the main product ginsenoside Rd and two sets of rare ginsenoside isomers with molecular weights of 980.56 and 962.55. HPLC-HRMS/MS analysis can provide sufficient information for their identification. The number and linkage sites of the glucose substituents are available in tandem mass spectra in the high *m*/*z* region. While the saturation of the olefin chain, the positions of the hydroxyl groups, double bonds, and other functional groups could be determined by the neutral loss investigation in lower *m*/*z* tandem mass spectra, thereby obtaining the characteristic structures of the aglycone, especially the olefin chain.

2.2. Time Course and Pathways of Ginsenoside Rb1 Biotransformation. Ginsenoside Rd is formed by the deglycosylation of only the outer glycosyl substituent at the C-20 position of ginsenoside Rb1. This biotransformation

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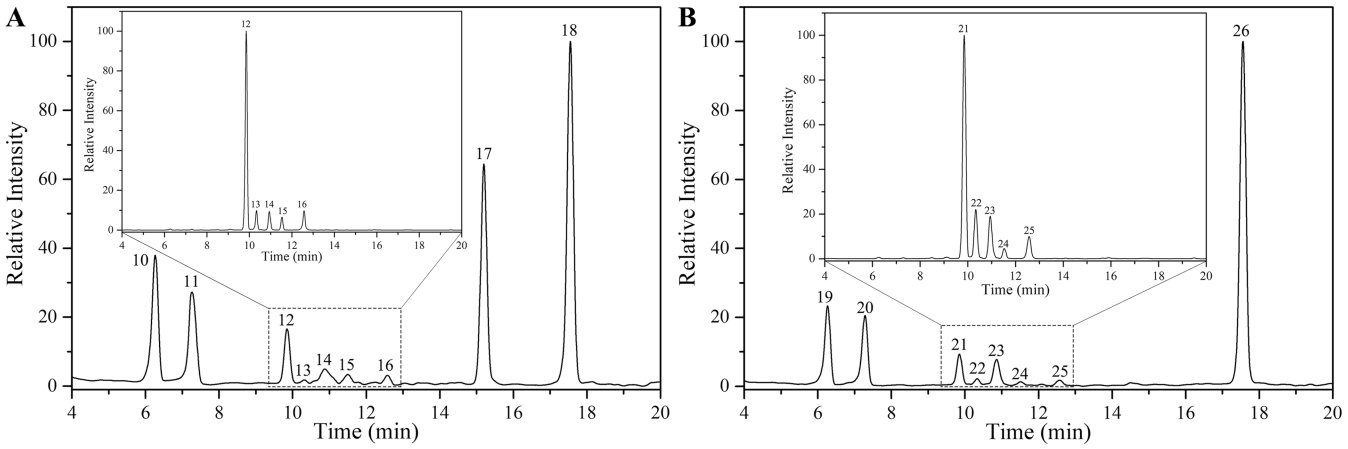

Figure 8. TICs of the biotransformation products of ginsenosides Rc (A) and Rd (B) generated by incubation with the culture medium of *I. lacteus*. Insets are the EICs of the ion at m/z 961.5392.

pathway is simple and straightforward. In contrast, the other byproducts require a multistep transformation of Rb1 for their formation. Time-course experiments were therefore carried out to elucidate the transformation pathways of these rare ginsenoside byproducts. As depicted in Figure 7, the transformation reaction almost finished after 14 days and the amounts of reactants and products remained constant over time. Ginsenoside Rb1 was rapidly transformed to Rd and compound **5** within 2 days after incubation with the culture medium of *I. lacteus*. On the 12th day, Rb1 was almost completely transformed and became undetectable. The amount of Rd gradually increased with the reaction time and accumulated to be the most abundant in the final products. The amount of compound **5** reached its maximum on the fourth day and then gradually decreased over time, indicating its further transformation to other products, which suggests that compound **5** was an intermediate product during the biotransformation. The other six byproducts accumulated gradually from the fourth day.

The order of appearance of the products suggested a reasonable pathway for the biotransformation of Rb1 as demonstrated in Scheme 1. In general, Rb1 is first transformed to Rd and compound **5** via deglycosylation at the C-20 position and hydroxylation at the C-22 position and then to other byproducts via hydroxylation at the C-23 position and transfer, hydrogenation, and cyclization of the double bond. The peak areas of compounds **1** and **2** are about 1 order of magnitude higher than those of the other byproducts, indicating that they tend to be more readily formed among the byproducts. The epimers, i.e., compounds **1** and **2** and compounds **4** and **7**, are found in similar amounts with the same variation trend, revealing the lack of stereoselectivity for the hydroxylation at the C-23 position and the cyclization at the C-22 position.

The biotransformation pathway of Rb1 by *I. lacteus* is quite different from that commonly reported, which is mainly deglycosylation accompanied by minor hydration and dehydration reactions.[20,32] *I. lacteus* can catalyze the transfer, hydrogenation, and cyclization of the double bond and the hydroxylation of the olefin chain to produce a variety of rare ginsenosides with novel structures. These transformation pathways of ginsenosides are extremely rare not only in biotransformation but also in chemical transformation where reaction conditions are much harsher. Although the yields of the transformed rare ginsenosides are not high, the biotransformation of ginsenosides using *I. lacteus* is still an effective approach to obtain structurally novel rare ginsenosides.

2.3. Verification of the Biotransformation Activity of *I. lacteus*.

To further verify the biotransformation activity of *I. lacteus*, ginsenosides Rc and Rd were used instead of Rb1 to incubate with the culture medium of *I. lacteus* under the same experimental conditions. The TIC of the biotransformation products of Rc is shown in Figure 8A, which is similar to that of Rb1 (Figure 1). Nine well-separated peaks are observed and defined as compounds **10**−**18**, whereas for Rd, eight peaks are defined sequentially as compounds **19**−**26**, as shown in Figure 8B. By comparison of the retention times, molecular weights, and tandem mass spectra with those of authentic standards, compound **17** was confidently identified as the reactant Rc, while compounds **18** and **26** were assigned as the product and reactant Rd, respectively. The retention times and fragment ions of compounds **10**−**16** and compounds **19**−**25** are correspondingly identical to those of compounds **1**−**7**, as shown in Figures 1 and 8, as well as in Tables S1−S3. This indicates that both Rc and Rd can be biotransformed by *I. lacteus* in the same manner as Rb1 to produce the seven rare ginsenoside byproducts mentioned above. It also suggests that the production of these seven rare ginsenosides is not limited to the use of Rb1 alone but can potentially take advantage of other PPD-type ginsenosides such as Rc and Rd.

Notably, the glycosyl substituent at the C-20 position of Rc is different from that of Rb1, consisting of a disaccharide moiety composed of arabinofuranose and glucose. *I. lacteus* is still able to deglycosylate this outer arabinofuranose substituent to produce Rd and seven rare ginsenosides. Correspondingly, Rd contains only a single glucose substituent at the C-20 position. *I. lacteus* does not deglycosylate this substituent and is therefore unable to biotransform Rd to ginsenoside Rg3. These results indicate that *I. lacteus* exhibits specificity for the outer glycosyl substituent at the C-20 position, demonstrating selective deglycosylation activity. Moreover, Rd was also transformed to the seven rare ginsenosides. It demonstrates that the transformation of Rb1 and Rc occurs initially by deglycosylation at the C-20 position to obtain the product Rd, and then further reactions such as the hydroxylation of the olefin chain generate the other seven

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rare ginsenosides. This observation corroborates the results of the time-course experiment.

3. CONCLUSIONS

In this study, ginsenoside Rb1 was biotransformed by incubation with the culture medium of *I. lacteus*, resulting in the main product ginsenoside Rd and two sets of isomeric ginsenoside byproducts. HPLC-HRMS/MS technique was used to rapidly identify the structures of these products and played a key role in their determination. It provided precise molecular weights and characteristic fragment ions of the transformed ginsenosides. By analyzing the structural possibilities of the neutral loss, we were able to deduce the number and position of the glycosyl substituents on the aglycone as well as the type and position of the functional groups on the olefin chain, which renders easy identification and discrimination of ginsenoside structural isomers. In contrast, it is not sensitive enough for the stereoconfiguration of chiral carbon atoms. Further confirmation of the absolute configuration of ginsenoside epimers in combination with the retention time is still needed.

Based on the structures of the transformation products, *I. lacteus* is found to be active in the hydrolysis of the outer glycosidic bond at the C-20 position, hydroxylation of the carbon atoms at the C-22 and C-23 positions, hydrogenation, transfer, and cyclization of the double bond at the C-24(25) position of ginsenoside Rb1. These enzymatic capabilities are not limited to the biotransformation of ginsenoside Rb1 but also extend to that of ginsenosides Rc and Rd, indicating the potential of *I. lacteus* for structural modification of PPD-type ginsenosides to produce a greater variety of rare ginsenosides. Similar activities have seldom been found in the biotransformation of ginsenosides. This is the first report of *I. lacteus* transformation activities in fungi. These activities are most likely the result of the synergistic effects of multiple enzymes. However, the key enzymes involved in the transformation remain unclear and need to be further obtained from a complex microbial system. In the future, the isolation and purification of these specific enzymes will greatly contribute to the targeted transformation of a wider variety of ginsenosides and the preparation of rare ginsenosides with novel structures in large quantities.

4. MATERIALS AND METHODS

4.1. Materials and Chemicals. Authentic standards of ginsenosides Rb1, Rc, and Rd with over 98% purity were acquired from Shanghai Yuanye Biological Technology Co., Ltd. (Shanghai, China). Potato dextrose agar (PDA) medium was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). HPLC-grade acetonitrile and formic acid were purchased from Tedia Company, Inc. (Fairfield, CT) and Thermo Fisher Scientific (Waltham, MA), respectively. Analytical-grade methanol and *n*-butanol were obtained from Beijing Chemical Industry Group Co., Ltd. (Beijing, China). Ultrapure water was collected from a Milli-Q water purification system (Millipore, Bedford). All other chemicals of analytical grade were purchased from commercial sources.

4.2. Microorganism and Growth Conditions. The fungal strain *I. lacteus* (CICC2555) was obtained from the China Center of Industrial Culture Collection (Beijing, China). It was stored at 4 °C on PDA slants and subcultured periodically. The freshly inoculated slants were incubated at 28 °C for 7 days before use. The mycelia were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of culture medium of which the component is 20.0 g/L of glucose, 15.0 g/L of peptone, 1.0 g/L of KH_2PO_3, and 1.0 g/L of $MgSO_4{\cdot}7H_2O$. The flasks were incubated on a thermostatic shaker at 28 °C with 160 rpm for 7 days. All media were sterilized at 121 °C for 30 min.

4.3. Biotransformation Procedures. Ginsenoside standards were dissolved in sterilized water and added to the culture medium at a final concentration of 0.5 mg/mL. The culture was then incubated for 14 days at 28 °C while being shaken at 180 rpm. Ginsenosides were extracted by sonification with an equal volume of water-saturated n-butanol three times for 30 min each. The n-butanol extract was then combined and rotary-evaporated to dryness. The residue was redissolved in methanol and filtered through a 0.22 μm membrane before HPLC-HRMS analysis.

4.4. HPLC-HRMS Conditions. An ACQUITY I-class UPLC system coupled with a SYNAPT G2-Si Q-TOF mass spectrometer (Waters Corp., Milford, MA) was used to identify the obtained ginsenosides in the biotransformation products. Chromatographic separation was achieved on an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) maintained at a temperature of 35 °C. 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) were used as mobile phases and delivered at a flow rate of 0.4 mL/min. The gradient elution was programmed as follows: 0−15 min, 15−30%B; 15:20 min, 30−35%B; 20:22 min, 35−90%B; 22:23 min, 90%B; 23−24 min, 90−15%B. The injection volume was 2 μL.

The elution was introduced into the high-resolution mass spectrometer via an electrospray ionization source interface operating at a source temperature of 120 °C, a desolvation temperature of 450 °C, a cone gas flow of 50 L/h, a desolvation gas flow of 1000 L/h, and a sampling cone voltage of 40 V. The mass spectrometer was operated in full scan mode to obtain the accurate mass of intact precursor ions and in MS/MS mode at a ramp collision energy of 20−60 V to obtain product ions of target precursor ions and corresponding accurate mass. Mass calibration was performed over the mass range of m/z 100−1500 using a 0.5 mmol/L sodium formate solution. Data were collected in negative acquisition mode from m/z 100 to 1500 with a scan time of 0.2 s. Leucine enkephalin at a concentration of 200 pg/mL was used as an external reference for mass correction. The solution was continuously infused at a flow rate of 10.0 μL/min during sample acquisition and acquired at 30 s intervals for 0.2 s.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c00837.

HRMS/MS spectrum from the $[M - H]^-$ ion at m/z 979.5480 (A) and magnified spectrum of the low m/z region (B) of compound **2** (Figure S1); HRMS/MS spectra from the $[M - H]^-$ ion at m/z 961.5392 of compounds **4** (A), **5** (B), **6** (C), and **7** (D) (Figure S2), and information on the biotransformation products and the major ions observed in the HRMS/MS spectra of ginsenosides Rb1 (Table S1), Rc (Table S2), and Rd (Table S3) (PDF)

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Notes

The authors declare no competing financial interest.

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