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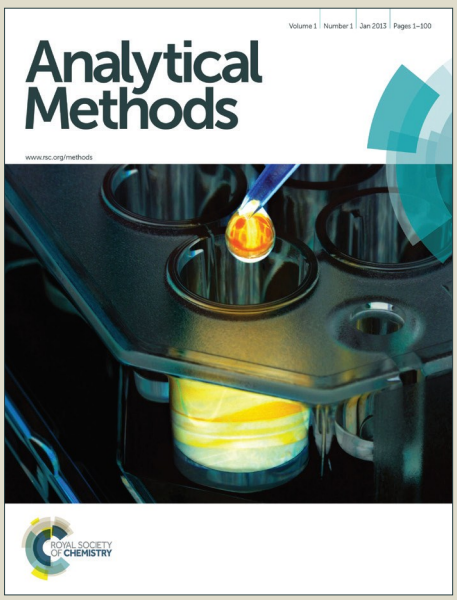

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A tractable method for the preparation of the ginsenoside compounds O and Mc1

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Abstract: The rare ginsenosides, compounds O and Mc1, were produced from the major ginsenosides Rb_2 and Rc by a *β*-glucosidase from *Esteya vermicola* via the pathways of Rb_2–compound O and Rc–compound Mc1. The biotransformation conditions for the compounds using *E. vermicola* CBS 115803 were established with the transformation temperature and time of 26 ºC and seven days, respectively, and the pH value of 5.5 at 130 rpm. The structure of the key metabolite was confirmed by MS analysis. The biotransformation yields for the two compounds were 96.7 and 95.2 %, respectively. A feasible method to recover the products was also developed, in which the products were purified from the fermentation broth, and the unreacted substrates were recovered from the mycelia. The present study provides a practical and potentially valuable approach that can be applied in preparations of compounds O and Mc1 without byproducts.

Keywords: *β*-glucosidase; ginsenosides Rb_2 and Rc; compounds O and Mc1; biotransformation.

Introduction

Ginseng (the *Panax genus*, Araliaceae) refers to a group of slowly growing plants with fleshy roots. [1] Among them, *Panax ginseng* C.A. Meyer is widely used in Asian countries as a traditional medicine[2]. The key components of ginseng are ginsenosides, and glycosides which contain an aglycone with a dammarane skeleton. The six major ginsenosides (Rg_1, Re, Rb_1, Rc, Rb_2 and Rd) constitute more than 80% of the total ginsenosides in Korean and American ginsengs[1,2]. The production of the minor ginsenosides staring from the major ginsenosides has been accomplished through physiochemical methods[3]. Recently, the enzymatic[4] and microbiological method[5] have attracted increasing attentions due to their higher conversion efficiency, fewer byproducts, superior environmental protection, and better stereospecificity. The four major protopanaxadiol-type ginsenosides, Rb_1, Rb_2, Rc, and Rd, are generally metabolized to compounds K, Y, and C[6,7]. These metabolites were investigated and showed *in vitro* and *in vivo* antitumor activities[8,9]. Although transformation pathways and identification of the above-mentioned ginsenosides have been reported,many pathways remained to be elucidated because of the diversity of enzymes. The biotransformation pathways of Rb_2 and Rc have been investigated[10,11], however, the resulted compounds O and Mc1 (Fig.1), and their intermediate metabolites (Fig.1), were rarely studied[12]. In the present study, the biotransformation pathways for the ginsenosides Rb_2 and Rc were described using a nematophagous fungus *Esteya vermicola*. The enzyme produced during the fermentation process individually converted effectively the ginsenosides Rb_2 and Rc to compounds O and Mc1 by selective hydrolysis of the terminal glucose moiety attached to C-3. The final converted metabolites are compounds O and Mc1, instead of compounds Y and Mc reported in the literatures[6,7]. To our knowledge, this is the first report for the preparation of compounds O and Mc1 using a biotransformation approach.

Experimental Section

Materials

The pure ginsenosides Rb_2 and Rc were purchased from Hongjiu Biotech Co. Ltd. (Jilin Province, China). The fungus *E. vermicola* CBS 115803 was obtained from Centraalbureauvoor Schimmelcultures (CBS), maintained in potato dextrose agar (PDA; Difco, NJ, USA) slants at 4 ºC. For experiments, the fungus was inoculated on PDA plate, and cultured at 26 ºC for 7 days.

The substrates, *p*-nitrophenyl-β-D-glucopyranosidase (*p*NPG), *p*NP-β-L-arabino-pyranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-L-fucopyranoside, *p*NP-α-L-fucopyranoside, as well as other disaccharides, were obtained from Sigma (St. Louis, MO, USA).

Assay of β-glucosidase activity

The method of β-glucosidase activity assay was according to the previously optimized one [21]. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate the equivalent of 1 μmol of p-nitrophenol per minute under the assay conditions. Enzyme activities against different substrates were determined by measuring the amounts of glucose released from these substrates[13].

Biotransformation pathway

150 mL of potato dextrose broth (PDB) was weighed exactly to collect spores of strain *Esteya vermicola* CBS 115803 by rinsing the potato dextrose agar (PDA) slant, then sub-cultured for 3 days at 26 ºC without shaking. After the day 3, 50 mg of ginsenosides Rb_2 and Rc were respectively weighed exactly and dissolved in the culture medium and incubated afterwards on a rotary shaker (130 rpm) at 26 ºC for 7 days.

Product recovery

After the above mentioned fermentation process for 7 days, the fermented liquid was collected and extracted three times with an equal volume of n-butanol, and the organic layers were combined and evaporated in vacuum to obtain the biotransformation products. The mycelia were treated using the same method as mentioned above; the unreacted substrates (Rb_2 and Rc) were recovered.

Structural identification

ESI-MS^n experiments were performed on a Finnigan LCQ ion-trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with a Finnigan electrospray ionization source, capable of analyzing ions up to *m/z* 2000. Samples were dissolved in methanol, and introduced into the ESI source by continuous infusion peak at a flow of 3 μL/min. The spray voltage was set at 5.0 kV and the capillary temperature was set at 200 °C. The isolation width for MS^2 was 2.0 Da, and the collision energy (%) was 20%.

Results and discussion

Substrate specificity of β-glucosidase from strain *Esteya vermicola* CBS 115803

The tests with various p-nitrophenyl glycosides indicated that the enzyme was greatly specific to β-D-glucoside (Table 1). Compared to its activity with that of *p*NPG (100%), the activities against other aryl-glycosides were quite less (others were not detected). The survey was performed with two disaccharides. The relative activity to compare with sophorose (β-(1-2)) was 92.9 %. But the activity against lactose (β-(1-4)) was not detected. Thus, the enzyme exhibited specific activity to β-(1-2) glucosidic linkage.

Biotransformation pathway of ginsenosides Rb_2 and Rc by strain *Esteya vermicola* CBS 115803

Based on the structures of ginsenoside Rb_2 and enzyme specificity produced by the strain, we can theoretically propose two pathways for the biotransformation of ginsenoside Rb_2(Fig. 2) and Rc (Fig. 3)by strain *Esteya vermicola* CBS 115803. It was obvious that the intermediate metabolites (compound O or compound Mc1) could be generated by sequentially hydrolyzing the two glucoses at C-3 position. The routine pathway is displayed on the left side, and only compound Y or compound Mc was obtained by directly hydrolyzing the two glucoses at C-3 position. In the present study, we determined the liquid fermentation process of strain *Esteya vermicola* CBS 115803 cultured with ginsenosides Rb_2 and Rc. The results revealed that the strain converted ginsenoside Rb_2 into ginsenoside compound O and Rc into compound Mc1 without further hydrolysis after seven-day culture. And this pathway was different from the proposed biotransformation pathways with the final metabolite of compound Y or compound Mc. The high conversion yields of compounds O and Mc1 were acquired, 96.7 % and 92.9 %, respectively.

Structural identification of the metabolites of ginsenosides Rb_2 and Rc by ESI MS

In order to confirm the conversion results, the metabolites of ginsenosides Rb_2 and Rc by strain *Esteya vermicola* CBS 115803 were identified by ESI-MS (Fig. 4). Based on the mass difference of 939 and 917 in Fig. 4a, it's deduced that the ion peak at *m/z* 939 is sodium adducted, and the ion peak at *m/z* 917 is protonated. In order to further confirm the structure, tandem mass spectrometric analysis was performed, as shown in Fig. 5a and b. The Domon and Costello nomenclature[14]has been employed throughout this work to define the fragment ions from the ginsenosides. The ions

peaks at *m/z* 335 and 627 are observed from the cleavage of C-O bond at C_{20} of the metabolite in Fig. 5a. The tandem mass spectrum of the ion peak at *m/z* 335 is displayed in Fig. 5b. The presences of the cross-ring cleavage ions peaks respectively at *m/z* 305, 275, and 245 indicate the 1-6 linkage for the disaccharide residue, according to the reports[15,16]. In addition, the observation of the ion peak at *m/z* 203 and the *m/z* value (335) of the disaccharide residue demonstrates, a pentose and a hexose, the compositions of the disaccharide residue. The obtained information in Fig. 5c is the same as that in Fig. 5b. However, the relative abundance of $Z_{0\alpha}$ ion peak at *m/z* 661 is very high. Subsequently, its tandem mass spectrometric analysis was performed (Fig. 5d). The observation of the ion peak at *m/z* 481 indicates the presence of a hexose residue at the C-3 position of the metabolite (compound O).

The ESI mass spectrum of the metabolite of ginsenoside Rc is similar to that of ginsenosides Rb_2, as shown in Fig. 4b. The detections of the ions peaks at *m/z* 939 ($[M+Na]^+$), *m/z* 955 ($[M+K]^+$), and *m/z* 973 ($[M+C_4H_9]^+$) provided the information of the molecular weight (MW = 916) of the metabolite. The tandem mass spectra are depicted in Fig. 6, which are similar to those of the metabolite of ginsenoside Rb_2.

The above results prove that the two metabolites are compounds O and Mc1, which indicated the good specifity of the enzyme.

As it is known, the efficiency of conversion and biotransformation pathways may differ greatly due to the diversity of the resident microflora between individuals. Therefore, a more uniform and targeted biological action may be achieved by obtaining specifically transformed ginsenosides. Few studies have reported the preparation processes of compounds O and Mc1 respectively by ginsenoside Rb_2 and ginsenoside Rc without further hydrolysis.

Here we have focused on understanding the transformation pathways of ginsenosides Rb_2 and Rc by a biocontrol fungus, *E. vermicola*. Generally, a strain might contain a series of enzyme, such as α-arabinopyranosidase, β-arabinopyranosidase, and β-glucopyranosidase. Theoretically, the three sugar moieties attached to ginsenoside Rb_2 or Rc could be hydrolyzed by various enzymes; namely, the outer and inner moieties attached at positions C-3 and C-20 could be hydrolyzed with various transformation pathways. However, in this study, the strain CBS115803 selectively hydrolyzed only one outer glucose at position C-3 for major ginsenosides Rb_2 and Rc. The

metabolism pathway could give a direct explanation for the final products of compounds O and Mc1. More interestingly, Rb_2- compound O and Rc- compound Mc1 appear to be the metabolism pathways for the long fermentation process with the strain CBS115803. It was different from the previous report [12] that the pathway was as follows: Rb_2- Compound O- F_2 and Rc- Compound Mc1- F_2 were obtained under long time reaction. It also differed from the other reports[13,17-19], in which, the pathways have been reported as Rb_2- Compound Y, Rb_2- Compound Y- Compound K, Rc- Compound Mc, and Rc- Compound Mc- Compound K.

In addition, during the fermentation process, only β-glucosidase was found from the strain CBS115803. Here it showed that the strain CBS115803 provided a potential resource for preparation of β-glucosidase. Compared with its activity against *p*NPG (100%), β-glucosidase against other aryl-glycosides were not able to be detected. Relatively high activity of sophorose (β-(1-2)) indicates that the β-glucosidase produced by *E.vermicola* possesses a specific activity on β-(1-2) linkage.

Intriguingly, in this study we also found a similar product recovery method with previous reports[20, 21]. The finally converted product can only be obtained in the fermented liquid and the rest of the substrate Rb_2 or Rc remained in the mycelia. It is quite convenient to purify the product and recover the substrate without further time-consuming process.

Conclusions

In conclusion, this is the first report on a biotransformation pathway for the preparations of compound O by Rb_2 and compound Mc1 by Rc without byproducts. The strain *Esteya vermicola* CBS115803 exhibited a tractable and specific bioconversion process. The results reported here may be significant to practical application. It offered a path to selectively produce alternative ginsenosides. The transformation enzyme from culture media of the strain *Esteya vermicola* CBS115803 will be isolated and purified in the near future. And further identify the enzyme system for large-scale productions of bioactive ginsenoside compounds O and Mc1.

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Microbial transformation of ginsenoside Rg3 to ginsenoside Rh2 by Esteya vermicola CNU 120806. World J Microbiol Biotechnol 28:1807-1811.

Table 1 Hydrolytic activity of β-glucosidase from strain CBS115803 on various substrates

Substrate	**Activity (%)[a]**
*p*NPG	100 ± 0.17
pNP-β-L-arabinopyranoside	ND
pNP-α-L-arabinopyranoside	ND
pNP-β-L-fucopyranoside	ND
pNP-α-L-fucopyranoside	ND
Sophorose	92.9
Lactose	ND

[a] Activity expressed relative to activity measured on *p*NPG (100%).

ND: activity is not detected by the analytical method used in this study. Data represent the means of three independent experiments.

Figure Legends

Fig.1. Chemical structures of the protopanaxadiol type saponins. glc: *β*-D-glucopyranosyl; arap: α-L-arabinopyranosyl; araf: α-L-arabinofuranosyl.

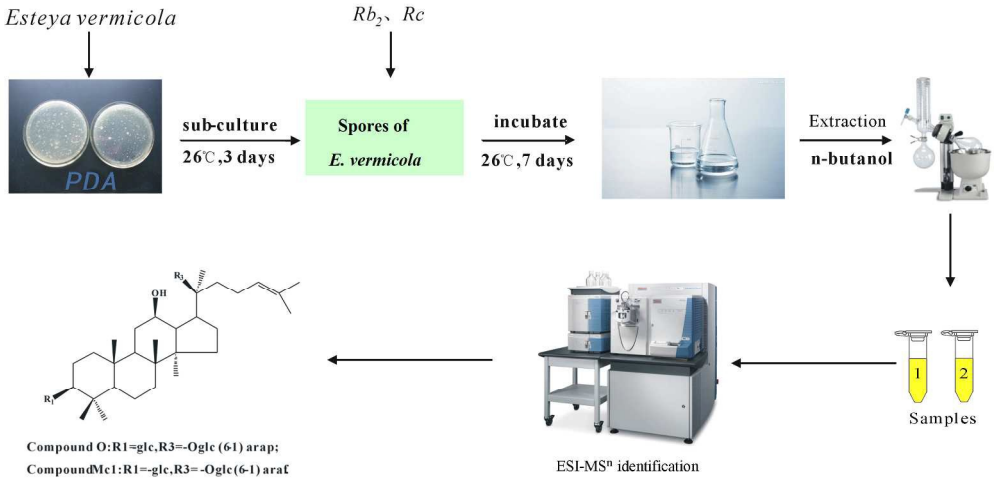
Fig.2. Proposed transformation pathways of Rb2 by the strain *Esteya vermicola* CBS 115803.

Fig.3. Proposed transformation pathways of Rc by the strain *Esteya vermicola* CBS 115803.

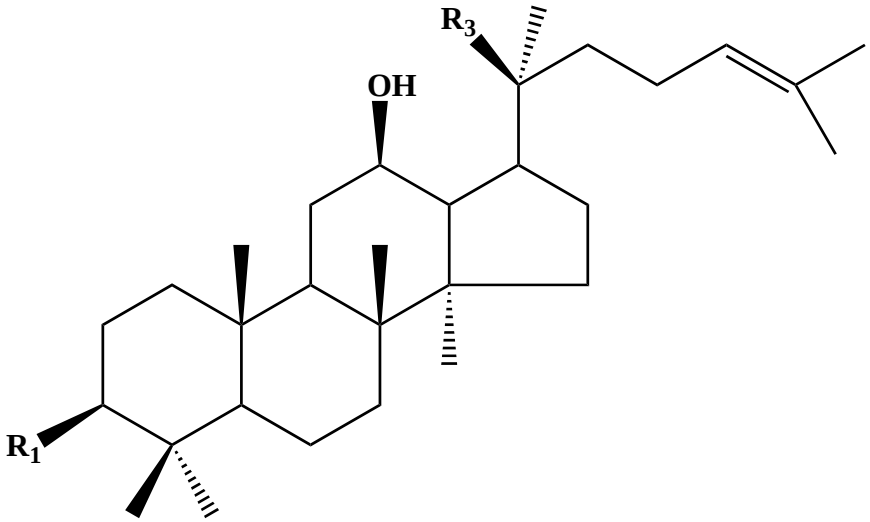
Fig. 4. The ESI mass spectra of metabolites of (a) ginsenosides Rb2 and (b) Rc by strain *Esteya vermicola* CBS 115803 in positive ion mode.

Fig.5. (a) ESI MS^2 and (b) ESI MS^3 (939→335) spectra of the sodiumated metabolite of ginsenoside Rb_2 in positive ion mode; (c) ESI MS^2 and (d) ESI MS^3 (973→661) spectra of the metabolite $[M + C_4H_9]^+$ of ginsenoside Rb_2 in positive ion mode.

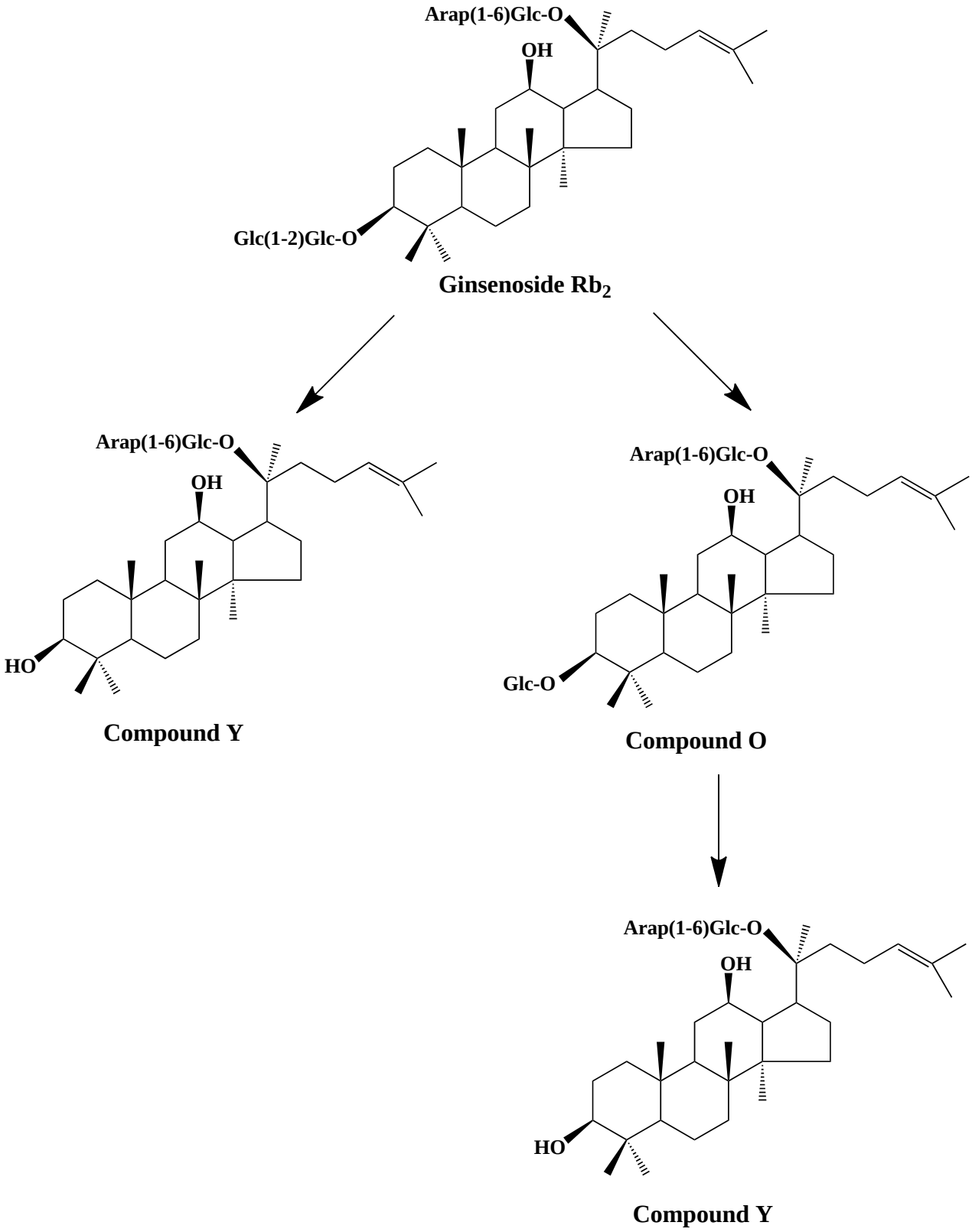
Fig.6. (a) ESI MS^2 and (b) ESI MS^3 (939→335) spectra of the sodiumated metabolite of ginsenoside Rc in positive ion mode; (c) ESI MS^2 and (d) ESI MS^3 (973→661) spectra of the metabolite $[M + C_4H_9]^+$ of ginsenoside Rc in positive ion mode.

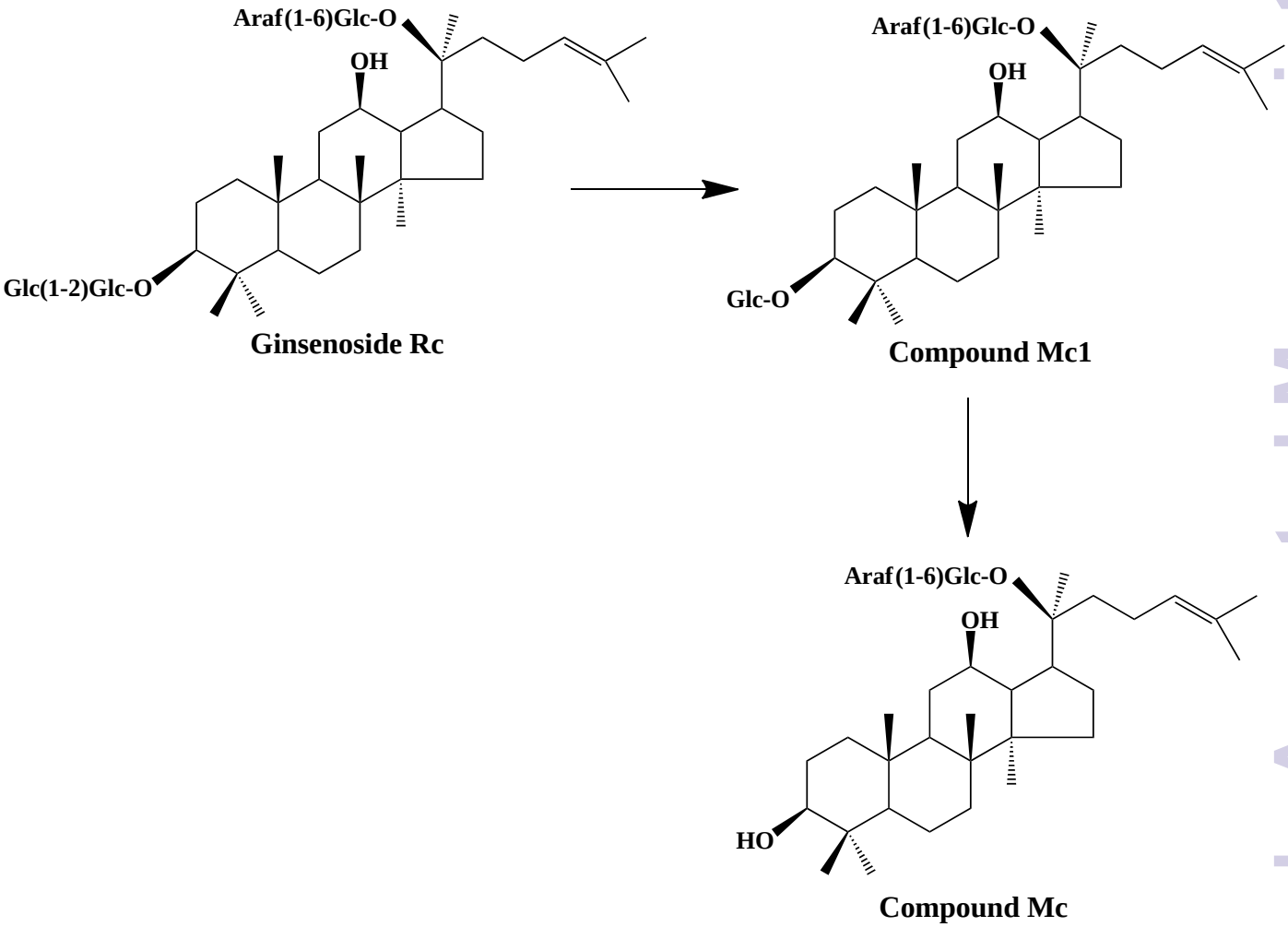


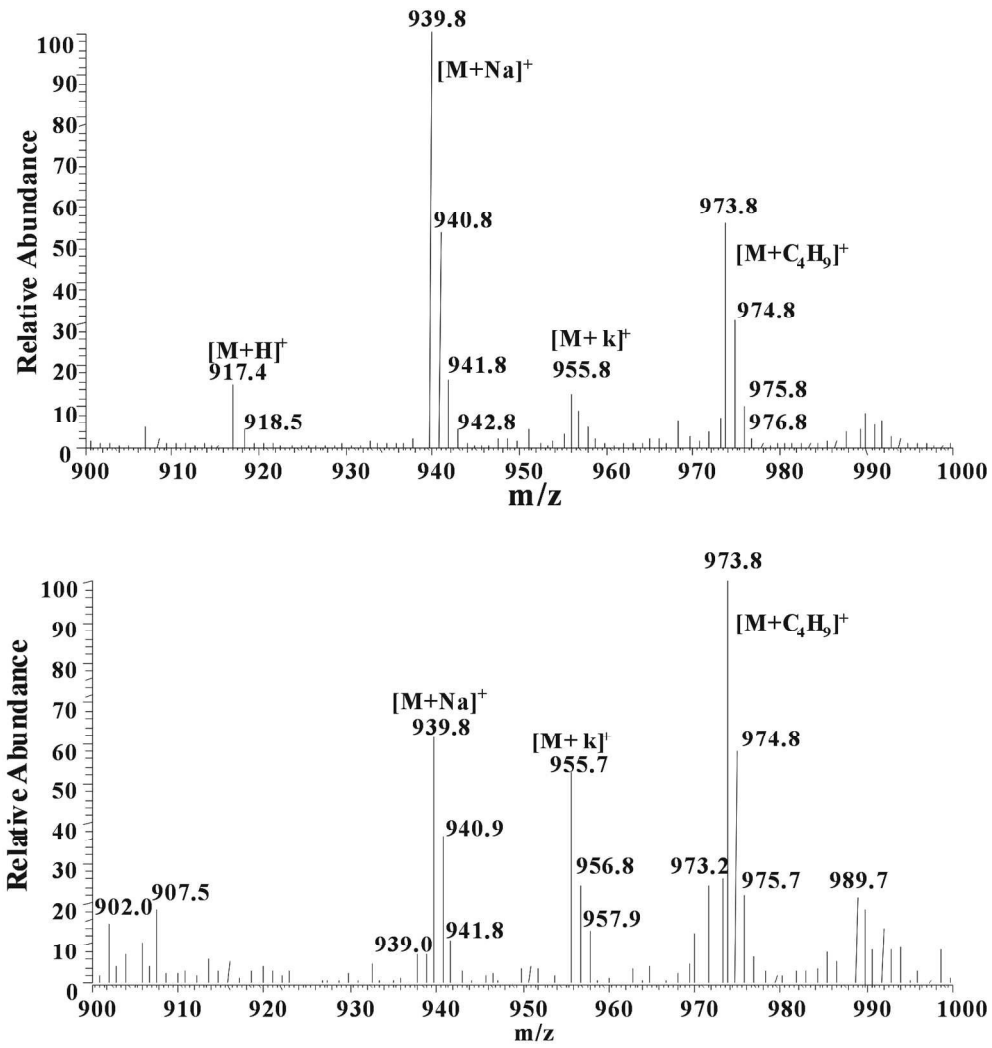
Graphical Abstract



Rb_2:R_1=-Oglc (2-1) glc,R_3=-Oglc (6-1) arap;
Rc:R_1=-Oglc (2-1) glc, R_3=-Oglc (6-1) araf;
Compound O:R_1= -glc,R_3= -Oglc (6-1) arap;
Compound Mc1:R_1= -glc,R_3= -Oglc (6-1) araf







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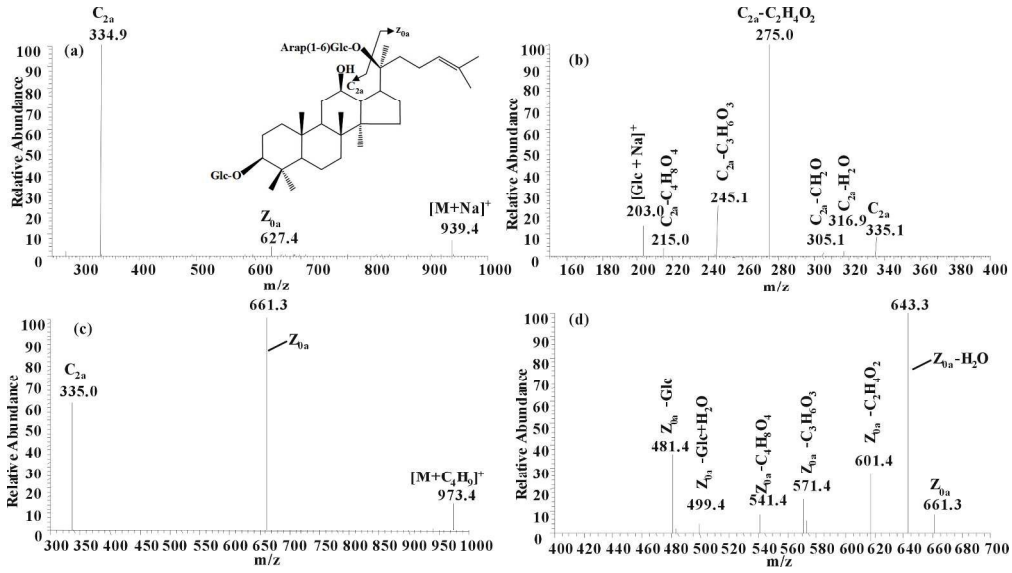

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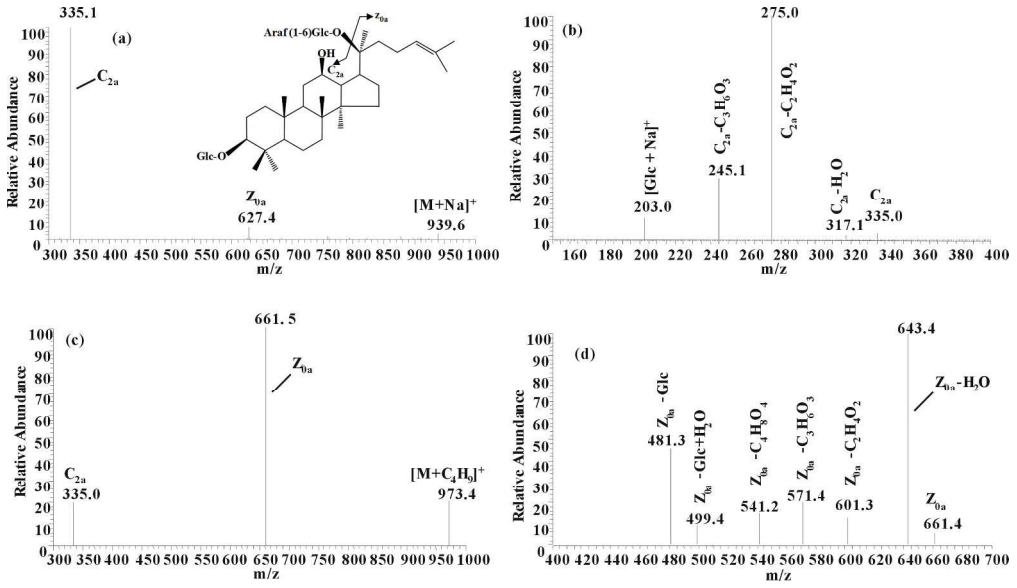

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