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Regioselective acylation of ginsenosides by Novozyme 435

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Abstract—Ginsenosides from *Panax* species were acylated regioselectively by Novozyme 435 with vinyl acetate as the acetyl donor in organic solvents to afford mono-acyl ginsenosides.

Ginseng (*Panax* spp.; Araliaceae) is one of the most important medicinal plants used in traditional Chinese medicine. It is claimed that ginseng has many beneficial pharmacological effects on human health, such as anti-tumour,[1] immunomodulatory and stimulatory effects on the central nervous system.[2,3] Ginseng saponins (ginsenosides) have since been identified to be the major bioactive ingredients of ginseng species.[2] Searching for novel natural products (e.g. ginsenosides) from plant species remains one of the principal ways to obtain bioactive lead compounds for drug discovery. To date, more than 40 different natural ginsenosides with many bioactivities have been isolated from the 13 *Panax* species.[4] However, it is a formidable task to search for novel ginsenosides from *Panax* spps. due to the tedious chromatographic procedures required for their purification.

Therefore, studies are now undertaken using microbial, chemical or enzymatic modification of natural glycosides.[5–9] Acylation of natural compounds could be important in the pharmaceutical applications of saponins since it provides a further dimension for the structural diversity of the natural saponins.[5,7–10] The acyl moieties could also be important for their bioactivity. For example, acylation of cholestane saponins was shown to increase the antitumour potency by 1000-fold.[11] Acylated saponins in low concentration also activate the metabolism of endothelial cells, which enhances the permeability of the blood vessel walls for better adsorption of the saponin into tissues.[12]

The major advantage of using enzymes, such as lipases, for modification, is their strong regioselective acylation properties on natural glycosides which may eliminate the protection/deprotection steps needed in the synthetic chemical route, and also the possibility to carry out reactions under mild conditions. Although lipase-catalyzed acylation is one of the most extensively studied enzymatic transformations, use of saponins as substrates has rarely been reported.[5,7,8] Novozyme 435 (Lipase B from *Candida antarctica*, a generous gift from Novozymes Australia Pty Ltd; enzyme activity expressed in Propyl Laurate units per gram (approx. 10,000 PLU/g)) has been shown to be an efficient catalyst for the regioselective acylation of the ginsenoside Rg1, Rb1 using vinyl acetate as the acyl donor in an organic solvent. Acylation occurs predominantly

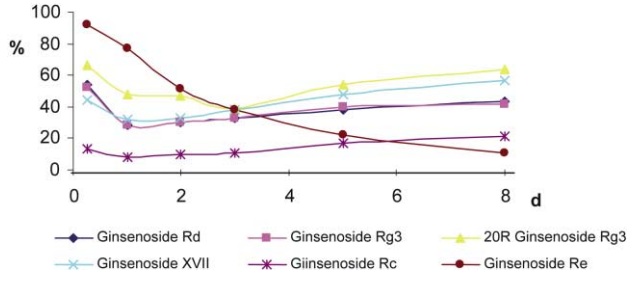

Figure 1. Acylation process of ginsenosides **1**–**3**, **5**–**6** and **8** catalyzed by Novozyme 435. (a) Data shows percentage of unreacted ginsenosides determined by UV monitoring of HPLC but not determined for **7** due to its low UV absorbance. (b) Only one acylated product for **1**–**3** and **5**; at least six products for **6**; three major products for **8** after a 2 day reaction and more than six products when the reaction proceeded for more than 2 days.

Keywords: ginsenosides; Novozyme 435; mono-acyl ginsenosides.
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on the primary hydroxyl groups (-CH_2OH) of glucose units.[5,7–9] Ready availability, low cost and knowledge of the catalytic mechanism of the enzyme make it an ideal catalyst in large-scale acylation of ginsenosides to increase their molecular diversity and potential applications. We carried out the acylation of different ginsenosides catalyzed by Novozyme 435 using vinyl acetate as the acyl donor. This paper reports the acylation results of eight ginsenosides as substrates.

Ginsenosides (**1**–**8**) (1–2 mg) were dissolved in 100 μl *t*-amyl alcohol or *t*-amyl alcohol: pyridine (10:1, v/v) in the presence of Novozyme 435 (3–5 mg) and vinyl acetate (50 μl) to determine the optimum reaction time and how many products were produced for each ginsenoside. The reaction process was monitored by TLC and HPLC. TLC and HPLC showed only one product was produced for ginsenosides Rd **1**, Rg3 **2**, 20*R* Rg3 **3**, Rh2 **4** and gypenoside XVII **5**. A single product with optimum yield was achieved after a 1 day reaction for ginsenosides Rd **1**, Rg3 **2**, gypenoside XVII **5** and a 3 day reaction for ginsenoside 20*R* Rg3 **3** (Fig. 1). For example, in the Novozyme 435 catalyzed acylation of ginsenoside Rd **1**, the yield of product **1a** was determined by HPLC as 46% after a 6 h reaction, 72% after a 1 day reaction, 70% after a 2 day reaction and 67% after a 3 day reaction (Fig. 1). These results show that hydrolysis occurs after longer reaction times for these ginsenosides. The acylation process of pseudoginsenoside F11 **7** was not determined due to its low UV absorbance, but two major products **7a** and **7b** in about 15 and 4% yield, respectively, were detected after a 2 day reaction. The acylated products were more complex for ginsenosides Rc **6**, Re **8**. At least six products were detected by HPLC for ginsenoside Rc **6** even after only a 6 h reaction. Three major products were detected for

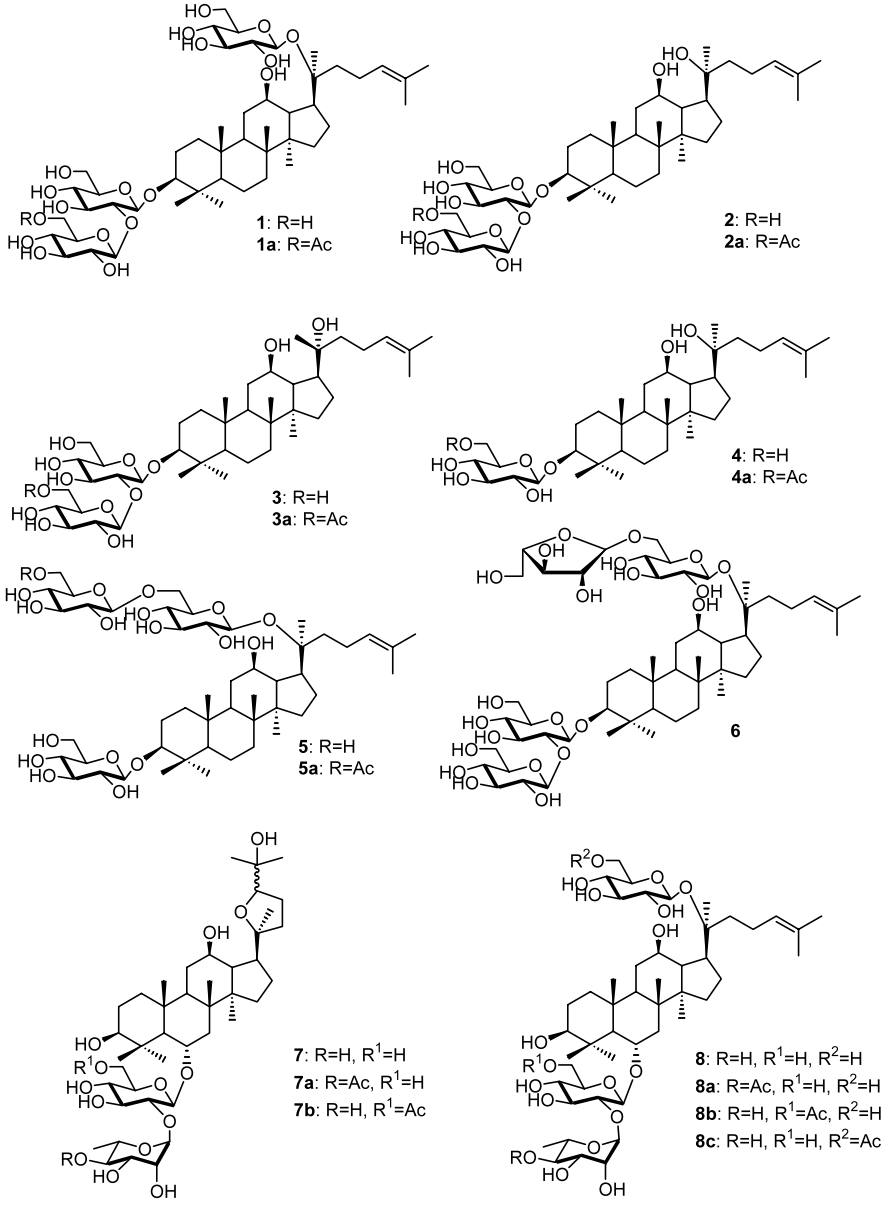

Figure 2. Ginsenosides and their acylated derivatives.

Table 1. Isolated yields of the acylated ginsenosides catalyzed by Novozyme 435

	1a	2a	3a	4a	5a	7a, 7b	8a, 8b, 8c
6 h					78%		
1 day	65%						
2 days						12%, 2.5%	11%, 4%, 4%
3 days		59%	66%	72%			

ginsenoside Re **8** after a 2 day reaction, while more products (six major products and more than six minor products) were detected with longer reaction times. Thus, the reaction time should be controlled in order to obtain the highest yield of the desired products. Another two lipases, lipase AP6 and Newlase (generous gifts from Amano Enzyme Inc. Nagoya, Japan) were employed to acylate three ginsenosides **6**–**8**, but only a trace of the acetylation products were detected.

Large scale acylation reactions using Novozyme 435 were then performed on **1** (100 mg), **2** (86 mg), **4** (78 mg), **7** (400 mg), **8** (360 mg) using similar reaction conditions as mentioned previously. For ginsenoside 20*R* Rg3 **3** (88 mg) and gypenoside XVII **5** (35 mg), the conditions were modified such that THF:DMF (4:1; v/v) was used as the dissolving solvent to enhance their solubility. The reactions were stopped after 6 h for gypenoside XVII **5**, 1 day for ginsenoside Rd **1**, 2 days for ginsenoside Re and pseudoginsenoside F11 **7**, 3 days for ginsenoside Rg3 **2** and ginsenoside 20*R* Rg3 **3**. The acetylated products were then isolated and purified by silica gel column chromatography using variable combinations of the mixture of $CHCl_3$, MeOH and H_2O as the eluent and Prep-HPLC using aqueous MeCN as the eluent. The structures of the products were then determined by MALDI-TOF MS and NMR spectra, including ^1H, ^{13}C, ^1H–^1H COSY, HMQC, HMBC and TOCSY. The structures of the acylated ginsenosides are summarized in Figure 2 and their isolated yields are shown in Table 1, respectively.[13]

For ginsenosides Rd **1**, Rg3 **2**, 20*R* Rg3 **3**, Rh2 **4** and gypenoside XVII **5**, acetylations only occurred on the primary OH-C(6) of the outer glucose unit with high yields, while pseudoginsenoside F11 **7** and ginsenoside Re **8** were primarily modified at OH-C(4) of the outer rhamnose unit with a yield of about 12% in addition to minor products at OH-C(6) of the glucose unit in yields of about 4–6%. The regioselectivity of Novozyme 435 was confirmed for the acylation of primary OH-C(6) groups of ginsenosides Rd **1**, Rg3 **2**, 20*R* Rg3 **3**, Rh2 **4** and gypenoside XVII **5**.[14] The low reactivity of the primary OH groups of glucose in pseudoginsenoside F11 **7** and ginsenoside Re **8** might be due to the steric hindrance caused by the terminal rhamnose affecting the interaction between glucose and lipase.

The 'upper' gentiobiose moiety linked at C-20 is reported to be more reactive than the 'lower' sophorose unit linked at C-3 in ginsenoside Rb1 for biocatalyzed acylation and galactosylation.[14] A similar situation is observed in gypenoside XVII **5** where acylation only occurred on the outer glucose unit linked to C-20, while the primary OH-C(6) of the outer glucose linked at C-3 of the aglycone was acylated in the case of ginsenoside Rd **1**. It seems that the side chain of the aglycone affects the acetylation of the single glucose linked at C-20 of the aglycone. In contrast to panaxatriol ginsenosides, the glucose moiety linked at C-20 in the panaxadiol ginsenosides is much less reactive as acetylation takes place preferentially at the oligosaccharide linked at C-6 for pseudoginsenoside F11 **7**, ginsenoside Re **8** and Rg1.[7]

In comparison to the fully assigned ^1H and ^{13}C NMR chemical shifts of the native ginsenosides lacking acetyl groups,[15–17] the acylation induced shifts (AIS) were diagnostic of the acylation position. From the assigned NMR data, acetylation caused deshielding of the α-carbon (C-6) of the glucose unit by 2–2.2 ppm, shielding of the β-carbon (C-5) of the glucose unit by 1.4–3.1 ppm, deshielding of the α-carbon (C-4) of the rhamnose unit by ~2 ppm, shielding β-carbons C-3 and C-5 of the rhamnose unit by 2.8–2.2 and 2.4 ppm, respectively. Meanwhile, deshielding of the α-methylene hydrogen of the glucose unit was ~0.47 ppm and the α-methine hydrogen of the rhamnose by ~1.50 ppm.

The acetylated ginsenosides are currently undergoing evaluation as potential anti-cancer agents.

Acknowledgements

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13. Selected analytic data: **1a**: MALDI-TOF MS: 1027.5 ($M+K^+$), 1011.6 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.1 (Me*C*OO), 131.0, 126.0, 106.2, 105.0, 98.4, 89.3, 84.4, 83.4, 79.3, 78.6, 78.4, 78.2, 78.0, 76.8, 75.4 (C-5″), 75.2, 71.6, 71.4, 71.1, 70.3, 64.8 (C-6″), 62.9, 62.8, 56.5, 51.8, 51.5, 50.2, 49.5, 40.1, 39.8, 39.2, 37.0, 36.1, 35.2, 31.0, 30.8, 28.1, 26.9, 26.7, 25.8, 23.3, 22.5, 21.0 (*Me*COO), 18.5, 17.8, 17.4, 16.5, 16.3, 16.0. **2a**: MALDI-TOF MS: 865.8 ($M+K^+$), 849.9 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.2 (Me*C*OO), 130.9, 126.4, 106.2, 105.0, 89.3, 84.4, 78.6, 78.2, 78.0, 76.8, 75.4 (C-5″), 73.2, 71.4, 71.1 (2×C), 64.9 (C-6″), 62.9, 56.5, 54.9, 51.8, 50.5, 48.6, 40.1, 39.9, 39.2, 37.0, 36.0, 35.3, 32.1, 31.5, 28.1 (2×C), 27.0, 26.9, 26.0, 23.1, 21.1 (*Me*COO), 18.6, 17.9, 17.1, 16.6, 16.5, 16.0. **3a**: MALDI-TOF MS: 865.8 ($M+K^+$), 849.8 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.2 (Me*C*OO), 130.9, 126.1, 106.3, 105.0, 89.2, 84.4, 78.6, 78.3, 78.0, 76.8, 75.4 (C-5″), 73.1, 71.4, 71.1 (2×C), 64.9 (C-6″), 62.9, 56.5, 51.9, 50.9, 50.5, 49.3, 43.3, 40.1, 39.8, 39.2, 37.0, 35.3, 32.2, 31.5, 28.1, 26.9, 26.7, 26.0, 22.9, 22.7, 21.0 (*Me*COO), 18.6, 17.8 (2×C), 16.6, 16.5, 15.9. **4a**: MALDI-TOF MS: 687.7 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 170.9 (Me*C*OO), 130.8, 126.4, 107.1, 89.3, 78.6, 75.6 (C-5′), 75.0, 73.0, 71.8, 71.1, 64.9 (C-6′), 56.5, 54.9, 51.8, 50.5, 48.6, 40.1, 39.7, 39.3, 37.1, 36.0, 35.2, 32.1, 31.4, 28.2 (2×C), 26.9, 26.8, 25.9, 23.1, 20.9 (*Me*COO), 18.6, 17.8, 17.1, 16.8, 16.4, 15.9. **5a**: MALDI-TOF MS: 1027.8 ($M+K^+$), 1011.9 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.1 (Me*C*OO), 131.1, 126.1, 107.0, 105.7, 98.2, 89.0, 83.6, 79.4, 78.8, 78.5, 78.2, 77.0, 75.9 (C-5‴), 75.2 (2×C), 75.0, 72.0, 71.6 (2×C), 70.8, 70.3, 64.9 (C-6‴), 63.2, 56.5, 51.7, 51.5, 50.3, 49.6, 40.1, 39.8, 39.3, 37.0, 36.3, 35.2, 30.9 (2×C), 28.2, 26.8 (2×C), 26.0, 23.3, 22.4, 21.0 (*Me*COO), 18.5, 18.1, 17.5, 16.9, 16.4, 16.1. **7a**: MALDI-TOF MS: 865.4 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.1 (Me*C*OO), 101.8 (2×C), 86.8, 85.7, 79.5, 78.8, 78.6 (2×C), 76.2 (C-4″), 74.4, 72.6, 72.4, 71.2, 70.5 (C-3″), 69.8, 67.1 (C-5″), 63.0, 61.2, 52.2, 50.3, 49.4, 48.3, 46.2, 41.2, 40.1, 39.7 (2×C), 32.9, 32.6, 32.3, 31.8, 28.9, 27.9, 27.6, 27.2, 27.1, 25.6, 21.3 (*Me*COO), 18.3, 18.2, 17.9, 17.6, 17.0. **7b**: MALDI-TOF MS: 865.6 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.0 (Me*C*OO), 102.2, 101.4, 86.8, 85.7, 79.1, 78.5, 78.4, 75.5 (C-5′), 74.4, 74.2, 72.5, 72.4 (2×C), 71.3, 70.5, 69.5, 65.0 (C-6′), 60.8, 52.3, 50.1, 49.5, 48.4, 46.2, 41.2, 40.0, 39.7, 39.5, 32.9, 32.5, 32.2, 31.7, 28.9, 27.8, 27.6, 27.2, 27.1, 25.6, 21.0 (*Me*COO), 18.8, 18.3, 17.8, 17.6, 16.9. **8a**: MALDI-TOF MS: 1011.8 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 171.0 (Me*C*OO), 131.1, 126.0, 101.8, 101.7, 98.4, 83.4, 79.5, 79.2, 78.8, 78.6, 78.5, 78.4, 76.2 (C-4″), 75.3, 74.8, 73.4, 72.6, 71.5, 70.4 (C-3″), 69.9, 67.1 (C-5″), 63.0, 62.8, 61.1, 51.9, 51.5, 49.7, 49.1, 46.1, 41.3, 40.1, 39.8, 39.4, 36.0, 32.2, 30.9 (2×C), 27.9, 26.7, 25.9, 23.4, 22.5, 21.2 (*Me*COO), 18.2, 17.9, 17.7, 17.5, 17.3, 17.2. **8b**: MALDI-TOF MS: 1027.8 ($M+K^+$), 1011.8 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 170.8 (Me*C*OO), 131.1, 126.0, 102.0 (2×C), 98.0, 83.4, 79.5, 79.2, 78.5, 78.4 (2×C), 75.0(C-5′), 74.8, 74.6, 74.2, 72.5, 72.3 (2×C), 71.6, 70.1, 69.4, 64.8 (C-6′), 63.0, 60.8, 51.8, 51.4, 49.6, 49.1, 46.0, 41.2, 40.0, 39.4 (2×C), 36.0, 32.2, 31.0, 30.8, 27.8, 26.7, 25.8, 23.1, 22.5, 20.8 (*Me*COO), 18.8, 17.8, 17.6, 17.5, 17.4, 17.3. **8c**: MALDI-TOF MS: 1027.6 ($M+K^+$), 1011.7 ($M+Na^+$); ^{13}C NMR (100 MHz, pyridine-d_5): 170.8 (Me*C*OO), 131.0, 126.0, 102.1, 101.4, 98.3, 83.3, 79.5, 79.3, 78.3 (3×C), 75.3 (C-5‴), 75.2, 74.6, 74.2, 72.5, 72.3 (2×C), 71.6, 70.3, 69.4, 65.0 (C-6‴), 63.2, 60.7, 51.7, 51.5, 49.6, 49.2, 46.1, 41.3, 39.9 (2×C), 39.4, 36.1, 32.1, 30.9 (2×C), 27.8, 26.6, 25.8, 23.3, 22.4, 20.9 (*Me*COO), 18.7, 17.7 (2×C), 17.5, 17.3 (2×C).
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