Efficient and highly regioselective acylation of andrographolide catalyzed by lipase in acetone†

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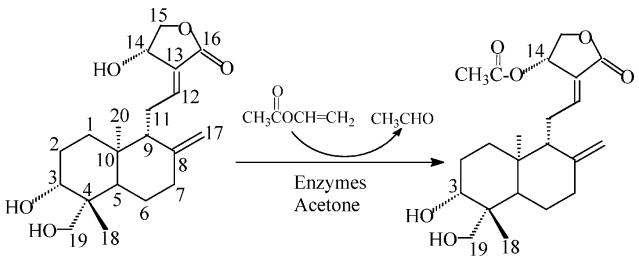
Andrographolide, a diterpene lactone from *Andrographis paniculata*, has multiple pharmacological activities. And its acetylated derivatives showed improved antitumour activities. Herein, a total of ten lipases and four proteases were tested for regioselective acylation(s) of andrographolide by vinyl acetate in acetone. Although the enzyme activity was markedly source-dependent, the highest conversion with better initial reaction rate was achieved with immobilized *Candida antarctica* lipase B (Novozym 435), which displayed higher operational stability. The regioselective acylation at the 14-hydroxyl of the lactone was established by a combination of spectroscopic methods including ^{1}H and ^{13}C NMR, DEPT, ESI-MS and FTIR spectra of the transformed product. The present work accommodated a feasible enzymatic approach for preparing andrographolide monoester, which possesses obvious advantages over the multi-step chemical process in terms of excellent regioselectivity, simplicity, environmental friendliness and mild reaction conditions.

Andrographis paniculata is an important herbal medicine traditionally used to treat a variety of diseases in some Southeast Asian countries such as China and India.[1–3] Andrographolide, the main and most pharmacologically active diterpene lactone of the plant (Scheme 1),[4,5] has been reported to have anti-cancer,[6–12] anti-inflammatory,[13–15] antiviral,[16] anti-allergic,[17]anti-diabetic[18] and immunostimulatory activities.[19–20] For the improvement of antitumour effects, andrographolide was also utilized as a starting material for synthesis of derivatives. Some acylated andrographolide analogs gave good antitumour actions against various cancer cell lines.[12,21] However, the selective monoacylation of andrographolide is difficult to achieve by means of conventional chemical approaches due to the similar reactivity of the hydroxyl groups in the molecule. Accordingly, the multi-step synthesis based on protection/deprotection reactions is required.[12,22] An alternative route is a biocatalytic process, which is not only facile and green, but could lead to selective modification of andrographolide. The discovery that enzymes are able to work in nonaqueous media, functioning in the reverse direction of that in nature, offer the opportunity to catalyze efficiently a wide variety of synthetic reactions.[23]



Scheme 1 Enzyme-catalyzed regioselective acylation of andrographolide with vinyl acetate in acetone.

Up to now, no study on enzymatic modification of andrographolide has been reported. We herein describe the first effort to carry out the regioselective acylation of andrographolide with vinyl acetate catalyzed by enzymes in acetone (Scheme 1) in order to develop a novel and efficient route for the preparation of a monoester of the lactone.

Different commercially available lipases and proteases were screened for their efficiency in the acylation of andrographolide with vinyl acetate in acetone. The results obtained are listed in Table 1. The andrographolide conversions and initial reaction rate (V_0) depended on the enzyme source. Four of the ten tested lipases showed substantial acylation activity. The best result was obtained when immobilized lipase from *Candida antarctica* (Novozym 435) was used as the biocatalyst, with >99% substrate conversion and 96.5% yield of the acylated product after a 5-hour treatment with the enzyme. And a moderate acylation activity was also observed using the lipases from *Thermomyces lanuginosus* (Lipozyme IM TL), *Rhizomucor miehei* (Lipozyme IM RM) and *Mucor miehei* (Lipozyme IM). Unfortunately, no protease of microbial or botanical origin had the desired acylation activity during the screening. Therefore, lipase Novozym 435 was selected for the subsequent study after scrutinizing the data tabulated in Table 1.

In order to characterize the structure of the acylated product afforded by the Novozym 435-catalyzed acetylation of andrographolide with vinyl acetate, the reaction was scaled up (andrographolide 350.0 mg) and the product was purified by silica gel chromatography (372.8 mg, 95.1%). The FT-IR spectroscopy of the product (Table 2) revealed the characteristic absorption bands at 3409 (ν_{OH}), 2865–2932 (ν_{CH}), 1679 ($\nu_{C=C}$) and 1751 cm^{-1} ($\nu_{C=O}$ of γ-lactone), also discernible in that of andrographolide. However, an extra IR signal of the product

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Table 1 Acylation of andrographolide with vinyl acetate catalyzed by various enzymes[a]

Entry	Enzyme[b]	Source[b]	V_0/mmol L^{-1} h^{-1}	C (%)[c]
1	Novozym 435	*Candida Antarctica*, type B	35.0	99.5
2	Lipozyme^{IM} TL	*Thermomyces lanuginosus*	14.1	51.0
3	Lipozyme^{IM} RM	*Rhizomucor miehei*	10.6	23.3
4	Lipozyme^{IM}	*Mucor miehei*	6.5	18.1
5	CRL	*Candida rugosa*, type VII	n.r.	—
6	PPL	Porcine pancreas	n.r.	—
7	CCL	*Candida cylindracea*	n.r.	—
8	Lipase A	*Aspergillus niger*	n.r.	—
9	Lipase M	*Mucor javanicus*	n.r.	—
10	Lipase AH	*Pseudomonas cepacia*	8.8	19.0
11	Alcalase	*Bacillus licheniforms*	n.r.	—
12	Neutrase	*Bacillus amyloliquefaciens*	n.r.	—
13	Protamex	*Bacillus subtilis*	n.r.	—
14	Papain	*Papaya latex*	n.r.	—
15	Control	—	n.r.	—

n.r.= no reaction.[a] The full experimental details are available as ESI.† The reaction conditions: 0.1 mmol andrographolide; 1.0 mmol vinyl acetate; 500 U enzyme; 5 ml acetone; $a_w = 0.07$; 45 °C; 150 rpm; 5 h. [b] The detail information about enzyme is available in ESI. [c] Andrographolide conversion to monoester (%), determined by HPLC.

Table 2 Main FT-IR spectral data for andrographolide and its ester

Compound	Wave number (cm^{-1})
Andrographolide	3407(ν_{OH}), 2868–2925(ν_{CH}), 1727(γ-lactone, $\nu_{C=O}$), 1674 ($\nu_{C=C}$), 1220/1032(ν_{C-O-C})
Product	3409(ν_{OH}), 2865–2932(ν_{CH}), 1770(acetyl, $\nu_{C=O}$), 1751(γ-lactone, $\nu_{C=O}$), 1679 ($\nu_{C=C}$), 1218/1209/1021(ν_{C-O-C})

Table 3 Chemical shifts (ppm) of andrographolide and 14-acetylandrographolide in CD_3OD[a]

Carbon	Andrographolide[b]	14-Acetylandrographolide[c]
1	38.2	38.1
2	29.0	29.0
3	80.9	80.8
4	43.7	43.7
5	56.4	56.2
6	25.2	25.1
7	38.9	38.8
8	148.8	148.9
9	57.4	57.2
10	39.9	39.9
11	25.7	26.3
12	149.4	151.0
13	**129.8**	**125.6**
14	**66.7**	**69.3**
15	**76.1**	**73.1**
16	172.6	171.9
17	109.2	108.8
18	23.4	23.4
19	65.0	64.9
20	15.5	15.5
Acetyl	—	171.3 (C=O); 20.7(–CH_3)

[a] ^{13}C NMR spectra are available in ESI. [b] Signals of ^{13}C NMR spectrum of andrographolide were assigned according to ref. 26–28. [c] Signals of ^{13}C NMR spectrum of 14-acetyl andrographolide were assigned according to ref. 12,22.

at 1770 cm^{-1}, ascribable to the acetyl carbonyl, indicated that andrographolide has been acylated.

High selectivity represents the most attractive characteristic of enzymatic reactions. It is well known that the acylation of a hydroxyl group results in a downfield shift of the peak corresponding to the *O*-acylated carbon atom and an upfield shift of the peak corresponding to the neighboring carbon atom.[24,25] The structure of the Novozym 435-catalyzed product was further characterized by ^{13}C NMR spectroscopy and it was found that, compared with andrographolide, the spectrum of the product exhibited two additional carbon signals at δ 171.3 (C=O) and 20.7 (CH_3), characteristic of an acetyl group (Table 3). Moreover, the C-14 (δ 66.7) of the andrographolide shifted downfield by 2.6 ppm and the resonance due to its neighboring carbon atoms C-13 (δ 129.8) and C-15 (δ 76.1) also showed an upfield shift of 4.2 and 3.0 ppm, respectively. The other carbons of the transformed product gave signals at positions almost identical with those of the starting substrate, suggesting the acylation occurred at 14-OH. DEPT 90 and 135 spectra data of the product also confirmed the above-mentioned conclusion.[29] And the aforementioned shift deviation could be well rationalized by the diamagnetic effect of the freshly introduced 14-*O*-acetyl group, which should have affected the chemical shifts of the neighbouring protons. As anticipated, in the ^1H NMR spectrum of the acylated product, H-14 signified at δ 6.01, shifting downfield by 1.00 ppm from that of andrographolide. Furthermore, the resonances arising from H-12 and H-15 moved slightly downfield without any change in the splitting pattern, whereas characteristic signals of hydrogens in the neighbourhood of 3- and 19-OH remained almost constant (Table 4). Thus, the Novozym 435-catalyzed acylation of andrographolide generated exclusively the 14-*O*-acetyl derivative in a regioselective manner. Moreover, the acetylation of andrographolide was further confirmed by the ESI-MS [m/z 392 (M^+)] and elemental analysis of the acetyl andrographolide (found: C, 67.47; H, 8.24. $C_{22}H_{32}O_6$ requires C, 67.34; H, 8.22%).

To further examine the potential of Novozym 435 for industrial production, its operational stability was investigated. After each batch reaction, the immobilized lipase was recovered

Table 4 Characteristic ^{1}H NMR spectral data of andrographolide and 14-acetyandrographolide in CD_3OD[a]

Hydrogen	Andrographolide	14-Acetylandrographolide
3	3.40 (1H, t, J = 8.1)	3.40(1H, t, J = 7.8)
12	6.85 (1H, td, J = 6.8, 1.7 Hz)	6.94(1H, td, J = 6.8, 1.7 Hz)
14	5.01 (1H, d, J = 6.1 Hz)	6.01(1H, d, J = 6.0 Hz)
15	4.46 (1H, dd, J = 10.2, 6.1 Hz)	4.57 (1H, dd, J = 11.1, 6.0 Hz)
	4.16 (1H, dd, J = 10.2, 2.2 Hz)	4.29 (1H, dd, J = 11.1, 1.9 Hz)
17	4.88 (1H, s); 4.67(1H, s)	4.88 (1H, s); 4.55 (1H, s)
19	3.35 (1H, d, J = 11.0 Hz)	3.36 (1H, d, J = 11.9 Hz)
	4.11 (1H, d, J = 11.0 Hz)	4.11 (1H, d, J = 11.1 Hz)
20	0.75 (3H, s)	0.72 (3H, s)
Acetyl	—	2.09(3H, s)

[a] ^{1}H NMR spectra are available in ESI.

by filtration and the next batch was carried out with fresh substrate. Novozym 435 displayed higher operational stability when andrographolide was used as feedstock and 90.5% of its original activity was maintained after being reused for 8 batches, highlighting the presumable cost-effectiveness of the enzyme.

Synthesis of 14-acetylandrographolide by chemical protection/deprotection reactions has been reported.[12,22] However, the reactions need more catalysts and reagents, and the overall yield is low (40.8%). In the present study, the biocatalyst Novozym 435 has been screened out to synthesize 14-*O*-acetylandrographolide with excellent regioselectivity in high yield (>95%). In particular, it avoids group protection procedures that usually need of use, and disposal of, eco-harming reagents. And the reaction conditions are mild with the follow-up purification accomplished in a much simplified manner. Since Novozym 435 is reusable for the purpose, the biocatalyst used herein can be thought to be cheap and eco-friendly. Furthermore, the advantage of acylating andrographolide enzymatically has been further improved with a rational selection of the solvent. Namely, the substitution of acetone for benzene or other toxic solvents as used elsewhere[12,22] has rendered the greener process.

Results described in this paper demonstrated that enzymatic acylation of the diterpene lactone is a novel and efficient route for the preparation of an andrographolide monoester. Generally, the enzymatic acylation in nonaqueous media as exemplified here for the regioselective esterification of andrographolide is a promising area that may be of value to acylate selectively other polyhydroxy organic compounds.

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29 DEPT 90 and 135 spectra are available in the ESI (Fig. 7)†.