Fitoterapia 80 (2009) 233–236

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Regioselective acylation of 3-O-angeloylingenol by *Candida antarctica* Lipase B

R.W. Teng [a,*], D. McManus [b,1], J. Aylward [c], S. Ogbourne [c], J. Johns [d], P. Parsons [d], A. Bacic [a]

[a] CRC for Bioproducts, School of Botany, The University of Melbourne, Victoria 3010, Australia
[b] CRC for Bioproducts, Tridan-Albright Ltd. and Wilson (Aust) Ltd. Partnership, Victoria 3013, Australia
[c] Peplin Ltd., 1 Breakfast Creek Rd, Newstead, Queensland 4006, Australia
[d] Queensland Institute of Medical Research, Herston, Queensland 4006, Australia

ARTICLE INFO

Article history:
Received 4 November 2008
Received in revised form 30 January 2009
Accepted 1 February 2009
Available online 8 February 2009

Keywords:
Candida antarctica Lipase B
Acylation
3-O-angeloylingenol

ABSTRACT

Acylation of 3-O-angeloylingenol (**1**) with vinyl acetate, vinyl decanoate and vinyl cinnamate, catalyzed by *Candida antarctica* Lipase B, was investigated. In each case, compound **1** was quantitatively and regioselectively acylated to afford a single product, 3-O-angeloyl-20-O-acetylingenol (**1a**), 3-O-angeloyl-20-O-decanoylingenol (**1b**) and 3-O-angeloyl-20-O-cinnamoylingenol (**1c**), respectively. The structures of the novel compounds **1b**–**1c** were determined by MS and NMR, and product **1a** by comparison of RP-HPLC and TLC with a standard. Compounds **1b**–**1c** induced a bipolar morphology of MM96L melanoma cells at a similar concentration as compound **1**, as well as having activity in inhibiting the growth of MM96L melanoma cells.

1. Introduction

Candida antarctica Lipase B (commercial name: *Novozyme 435*) is a lipase which has proven to be a highly regio- and stereo-selective enzyme in both hydrolysis [1] and synthesis [2]. This enzyme has been used to acylate a variety of substrates, including many classes of natural products [3]. We successfully used *C. antarctica* Lipase B for the regio- and stereo-selective acylation of ginsenosides [4], iridoid glycosides, a flavanoid glycoside and simple phenolic compounds [5].

3-O-angeloylingenol (**1**) was isolated from *Euphorbia antiquorum* and *E. peplus*, the extracts of which have been used in the treatment of a number of conditions, such as warts, corns, waxy growth and skin cancer [6]. First characterized as a skin irritant and a toxic *Euphorbia* factor, An1 [7], 3-O-angeloylingenol (**1**) was thereafter shown to induce *in vitro* apoptosis of melanoma cells [8] and leukemia cells [9]. This compound showed potential as a novel topical chemotherapeutic agent for the treatment of skin cancer [10] and is being developed as a topical therapy for actinic keratosis and superficial basal cell carcinoma (www.peplin.com).

In this study, 3-O-angeloylingenol (**1**) was acylated with vinyl acetate, vinyl decanoate and vinyl cinnamate in the presence of *C. antarctica* Lipase B. The acylated products **1a**–**1c** were isolated, purified and their structures determined by MS and NMR (Fig. 1). The activity of compounds **1b**–**1c** in inhibiting the growth and inducing a bipolar morphology of MM96L melanoma cells was assessed.

2. Experimental

2.1. General

Vinyl acetate, vinyl decanoate, vinyl cinnamate and 2-methylbutan-2-ol were purchased from Sigma. *C. antarctica* Lipase B (commercial name: Novozyme 435), immobilized on acrylic resin, was a kind gift from Novozymes Australia Pty Ltd. RPMI 1640 media were purchased from Life Technologies, USA, fetal calf serum and 96-well tissue culture plates from CSL Biosciences, Australia.

* Corresponding author. Current address: School of Biochemistry and Molecular Biology, Australian National University, ACT 0200, Australia. Tel.: +61 2 6125 0640; fax: +61 2 6125 0313.
E-mail address: tengrongwei@hotmail.com (R.W. Teng).
[1] Current address: Nutrifina Pty Ltd., 103 Albert St, Port Melbourne, Victoria 3207, Australia.

0367-326X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2009.02.001

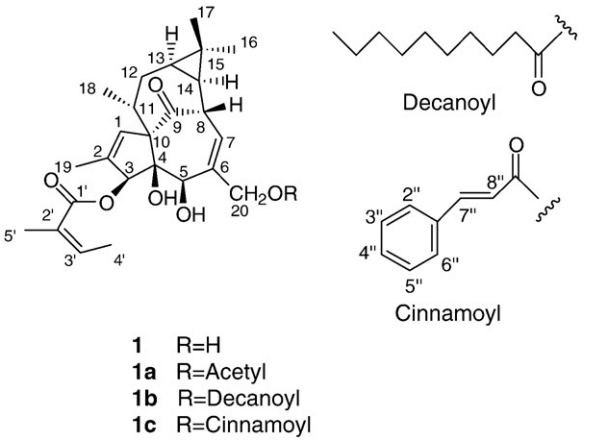

Fig. 1. Structures of 3-O-angeloylingenol (**1**) and its acylated derivatives (**1a**–**1c**).

Standards of 3-O-angeloylingenol and 3-O-angeloyl-20-O-acetylingenol were from Peplin Ltd., Australia. Pre-coated thin layer chromatogram (TLC) plates (silica gel 60 F_{254}, 0.25 mm) were purchased from Merck.

3-O-angeloylingenol (**1**) and its acylated products were separated on silica gel TLC using toluene:acetone (4:1, v/v) as a solvent system. After the TLC plate was dried, TLC plates were dipped into 5% (v/v) H_2SO_4 (in ethanol) followed by heating using a hair drier. 3-O-angeloylingenol (**1**) and its acylated products appeared as yellow coloured spots.

RP-HPLC was carried out on a Beckman Gold-126 (Beckman Coulter, Inc., USA) equipped with a variable wavelength UV monitor. An analytical Luna C18(2) HPLC column (5 µm, 150×4.6 mm) and a semi-preparative Luna C18(2) HPLC column (10 µm, 250×10 mm), purchased from Phenomenex Australia Pty Ltd., were used. RP-HPLC was performed with binary mixtures of 70% (v/v) MeOH in Milli-Q H_2O (A) and 100% MeOH (B) with a gradient: 0–10 min 0–50% (v/v) B, 10–35 min 50–83% B, then 35–40 min 83–100% B at a flow rate of 0.5 and 2.8 ml/min for analytical and preparative scale, respectively. Elution was monitored with UV detection at 210 and 230 nm.

^{1}H NMR were performed at room temp on a Varian Unityplus-400, at 400 MHz (^{1}H); δ in ppm relative to solvent signal of $CDCl_3$ (δ_H 7.24). MALDI-TOF-MS was performed on a Voyager-DE STR biospectrometry workstation (Perspective Biosystems) using α-cyano-4-hydroxycinnamic acid as a matrix as previously described [5].

2.2. Acylation of compound **1** with C. antarctica Lipase B

In small-scale experiments, compound **1** (approx. 0.3–0.6 mg) was dissolved in 2-methylbutan-2-ol (80–150 µl) and then *C. antarctica* Lipase B (3 mg) and acyl donors (50 µl) were added. Three separate incubations were performed, one for each acyl donor (vinyl acetate, vinyl decanoate or vinyl cinnamate). Blank controls were carried out for each acyl donor with solutions lacking *C. antarctica* Lipase B. The resulting mixtures were then incubated at 37 °C on a gyratory shaker at 250 rpm. A 10 µl of reaction solution was taken from the mixture and analysed by TLC and RP-HPLC to monitor reaction process. After substrate **1** was completely consumed (7 h for reaction with vinyl acetate, 1 d for vinyl decanoate and 7 d for vinyl cinnamate), the reaction was terminated by filtering off the enzyme through a filter paper. The filtrate was dried in a spin-vacuum prior to analysis.

To scale up, compound **1** (3 mg) was dissolved in 2-methylbutan-2-ol (0.5 ml). To each of two separate solutions, was added *C. antarctica* Lipase B (15 mg) and the acyl donors (180 µl), either vinyl decanoate or vinyl cinnamate, respectively. The reactions were terminated after 1 d for reaction with vinyl decanoate and after 7 d with vinyl cinnamate.

2.3. Isolation of acylated products

The dry residues were dissolved in MeOH (~1 ml) and then purified by preparative RP-HPLC to afford compounds **1b** and **1c**. The isolated yields of the acylated products from the scaled-up reactions were >90% for both compounds **1b** and **1c**.

2.4. MS and NMR data of acylated products

3-O-angeloyl-20-O-acetylingenol (**1a**). MALDI-TOF-MS ($C_{27}H_{38}O_7$; 474.59): *m/z* 473.11 $[M+H]^+$, 495.09 $[M+Na]^+$.

3-O-angeloyl-20-O-decanoylingenol (**1b**). MALDI-TOF-MS ($C_{35}H_{52}O_7$; 584.37): *m/z* 607.36 $[M+Na]^+$. ^{1}H NMR (400 MHz, δ in $CDCl_3$, *J* in Hz): δ 6.16 (1H, *qq*, *J* = 1.6, 7.2 Hz, H-3′), 6.12 (1H, br*d*, *J* = 4.0 Hz, H-7), 6.05 (1H, *q*, *J* = 1.6 Hz, H-1), 5.56 (1H, *s*, H-3), 4.79 (1H, *d*, *J* = 12.8 Hz, H-20a), 4.48 (1H, *d*, *J* = 12.8 Hz, H-20b), 4.12 (1H, *dd*, *J* = 4.4, 11.6 Hz, H-8), 3.88 (1H, *s*, H-5), 2.51 (1H, *m*, H-11), 2.30 (2H, *t*, *J* = 7.6, Me$(CH_2)_7CH_2$CO), 2.26 (1H, *ddd*, *J* = 2.8, 8.8, 15.6 Hz, H-12β), 2.03 (3H, *dq*, *J* = 7.2, 1.6 Hz, H-4′), 1.93 (3H, *d*, *J* = 1.6 Hz, H-5′), 1.81 (3H, *d*, *J* = 1.2, H-19), 1.76 (1H, *ddd*, *J* = 6.0, 11.6, 15.6 Hz, H = 12α), 1.25–1.29 (14H, *m*, Me$(CH_2)_7$CH$_2$CO), 1.09 (3H, *s*, H-17), 1.06 (3H, *s*, H-16), 0.98 (3H, *d*, *J* = 6.8 Hz, H-18), 0.94 (1H, *t*, *J* = 6.8 Hz, H-14), 0.88 (3H, *t*, *J* = 6.8 Hz, *Me*$(CH_2)_7CH_2$CO), 0.70 (1H, *dt*, *J* = 6.4, 8.4 Hz, H-13).

3-O-angeloyl-20-O-cinnamoylingenol (**1c**), MALDI-TOF-MS ($C_{34}H_{40}O_7$; 560.68): *m/z* 583.56 $[M+Na]^+$. ^{1}H NMR (400 MHz, δ in $CDCl_3$, *J* in Hz): δ 7.69 (1H, *d*, *J* = 16.0 Hz, H-7″), 7.37–7.55 (5H, *m*, H-2″ to H-6″), 6.45 (1H, *d*, *J* = 16.0 Hz, H-8″), 6.16 (1H, *qq*, *J* = 1.6, 7.2 Hz, H-3′), 6.18 (1H, br*d*, *J* = 5.2 Hz, H-7), 6.06 (1H, *q*, *J* = 1.2 Hz, H-1), 5.59 (1H, *s*, H-3), 4.95 (1H, *d*, *J* = 12.8 Hz, H-20a), 4.62 (1H, *d*, *J* = 12.8 Hz, H-20b), 4.14 (1H, *dd*, *J* = 4.0, 12.0 Hz, H-8), 3.94 (1H, *s*, H-5), 2.53 (1H, *m*, H-11), 2.28 (1H, *ddd*, *J* = 2.8, 8.8, 16.0 Hz, H-12β), 2.03 (3H, *dq*, *J* = 7.2, 1.6 Hz, H-4′), 1.93 (3H, br*s*, H-5′), 1.81 (3H, *d*, *J* = 1.2, H-19), 1.77 (1H, *ddd*, *J* = 6.0, 11.6, 16.0 Hz, H = 12α), 1.09 (3H, *s*, H-17), 1.06 (3H, *s*, H-16), 0.98 (3H, *d*, *J* = 7.2 Hz, H-18), 0.94 (1H, *t*, *J* = 7.6 Hz, H-14), 0.70 (1H, *dt*, *J* = 6.4, 7.6 Hz, H-13).

2.5. Bioassay

Compounds **1**, **1b** and **1c** were collected from RP-HPLC elutes when the 10 µl reaction solutions withdrawn during the small-scale acylation experiments were analysed by RP-HPLC. Approximately equal amounts were dried by vacuum centrifugation and dissolved in 50 µl of acetone. Control experiment was carried out using 50 µl acetone. The solutions were diluted at a series of 10-fold by complete cell culture

medium (RPMI 1640, supplemented with 10% (v/v) fetal calf serum.

One μL of the solution was added to a single well of a 96-well tissue culture plate containing 99 μl of complete cell culture medium and approximately 3000 MM96L melanoma cells which had been growing for 24 h . Assays for each dilution were conducted in duplicate.

Two days after treatment, the cells were observed for induction of a bipolar morphology, which indicated differentiation activity, in comparison to the poly-dentritic control. Five days after treatment, the cells were washed with phosphate-buffered saline, fixed in ethanol and then the total protein, an indicator of growth inhibition activity, was determined using sulforhodamine B [11].

3. Results and discussion

3-O-angeloylingenol (**1**) was reacted with either vinyl acetate, vinyl decanoate or vinyl cinnamate in the presence of *C. antarctica* Lipase B. The reactions were monitored by TLC and HPLC. Small scale experiments indicated that 3-O-angeloylingenol (**1**) was quantitatively acylated into a single product in the presence of each of the three acyl donors. To generate sufficient amounts of products for structural determination, the reactions were scaled-up and the acylated products were then purified by preparative RP-HPLC. The structures of acylated products were determined by MS and ^{1}H NMR. The results showed that acylations occurred only at the primary hydroxyl –OH(C-20) to afford 3-O-angeoyl-20-O-acetylingenol (**1a**), 3-O-angeloyl-20-O-decanoylingenol (**1b**) and 3-O-angeloyl-20-O-cinnamoylingenol (**1c**), respectively. The structures of compounds **1b** and **1c** have not been previously reported.

In the control reactions, no product formation was detected by either TLC or RP-HPLC. When reacted with vinyl acetate in the presence of *C. antarctica* Lipase B, 3-O-angeloylingenol (**1**) was completely transformed into a single product (compound **1a**) after only a 6 h incubation, as indicated by the disappearance of the spot of 3-O-angeloylingenol (**1**) (relative shift (Rf): 0.2) and appearance of a new spot (Rf: 0.6) on TLC. The result was further confirmed by RP-HPLC where a new peak for compound **1a** (retention time (Rt): 29 min) appeared and the substrate, 3-O-angeloylingenol (**1**) (Rt: 27 min) disappeared.

Reaction of 3-O-angeloylingenol (**1**) with vinyl decanoate in the presence of *C. antarctica* Lipase B was also rapid. Approximately 90% of 3-O-angeloylingenol (**1**) was consumed in 6 h and completely converted into compound **1b** in 1 d (**1b**, Rt: 48 min in RP-HPLC). Reaction of 3-O-angeloylingenol (**1**) with vinyl cinnamate was much slower than with vinyl acetate or vinyl decanoate (Fig. 2), presumably due primarily to the steric hindrance of the bulky phenyl group. The results of RP-HPLC fractionation indicated that 45% of 3-O-angeloylingenol (**1**) was transformed into compound **1c** (Rt: 37 min) in a 1 d reaction, 90% after a 3 d reaction, and complete conversion after 7 d.

The product **1a** was purified by preparative RP-HPLC and its structure was determined as 3-O-angeoyl-20-O-acetylingenol (**1a**), an –OH(C20) acetylated product, by co-elution on TLC and RP-HPLC with a 3-O-angeoyl-20-O-acetylingenol standard. This suggested that the primary

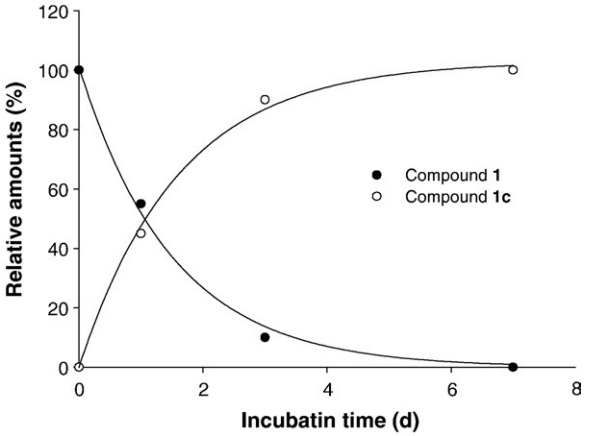

Fig. 2. Acylation kinetics of the conversion of 3-O-angeloylingenol (**1**) into compound **1c** using vinyl cinnamate in the presence of *C. antarctica* Lipase B.

hydroxyl group, –OH(C20), is the most reactive hydroxyl group in compound **1** (Fig. 1) and that the acylated products, **1b** and **1c**, should be the –OH(C20) acylated derivatives of 3-O-angeloylingenol (**1**).

In order to confirm the structures of compounds **1b** and **1c**, reactions were scaled up to generate sufficient quantities of **1b**–**1c**. The MALDI-TOF MS spectrum of compounds **1b** and **1c** showed quasi-molecular ions at m/z 607.36 $[M+Na]^+$ and 583.56 $[M+Na]^+$, respectively, which were 154 amu and 130 amu greater than the substrate 3-O-angeloylingenol (**1**) [10]. These increased molecular weights corresponded to an additional decanoyl and cinnamoyl group, respectively, which was confirmed by ^{1}H NMR of compounds **1b** and **1c** showing the additional ^{1}H signals for the decanoyl and cinnamoyl groups, respectively [4,5]. Moreover, compared with ^{1}H NMR data of compound **1** [12], the H-20 resonance of **1b** and **1c** were shifted downfield by 0.45 and 0.80 ppm, respectively, typical of acylation induced shifts [4,5]. Therefore, the structures of compounds **1b** and **1c** were deduced to be 3-O-angeloyl-20-O-decanoylingenol (**1b**) and 3-O-angeloyl-20-O-cinnamoylingenol (**1c**), respectively (Fig. 1).

Acylation of compound **1**, catalyzed by *C. antarctica* Lipase B, afforded a quantitatively single mono-acylated product. In addition, acylation occurred only at –OH (C20) while the other two hydroxyl groups were un-reacted. This degree of regioselectivity would be technically challenging for a non-enzymatically based acylation reaction.

Compounds **1** and **1b**–**1c** were dissolved in acetone and diluted in complete cell culture medium by a series of 10-fold dilutions for bioassay. All three compounds, in addition to compound **1a** assayed previously by Peplin Ltd. (data not published), showed similar levels of activity at inducing a bipolar morphology of the MM96L melanoma cells at approximately 1 ng/ml (a dilution of 10^5). All four compounds **1** and 1a–1c, also showed growth inhibitory activity against the MM96L melanoma cells. Whilst the results were not quantified, these data are interesting as differentiation and growth inhibition of mammalian cells are commonly associated with protein kinase C (PKC) activity [13,14] and acylation of ingenol esters at C20 would be expected to afford inactivity. These compounds could therefore prove useful in the elucidation of

PKC binding and activation kinetics. However, it is possible that cell mediated hydrolysis of compounds **1a**–**1c** yielded compound **1**, resulting in the observed positive biological assay results. Given the potential of compound **1** as an anti-cancer agent, compounds **1a**–**1c** may be interesting novel targets for product development. The bioassays indicate that acylation of compound **1** appear not to improve its biological efficacy.

Acknowledgements

This work was funded by a grant from the Australian Government to the CRC for Bioproducts. We wish to thank Novozymes Australia Pty Ltd. for providing Novozyme 435, and Drs. T. K. Lim and Frances Separovic, School of Chemistry, The University of Melbourne, Australia, for access to the NMR spectrometer. We also thank Dr Ming-Long Liao, CRC for Bioproducts, The University of Melbourne, Australia, for critical comments.

References

[1] (a) Brunet C, Zarevucka M, Zdenek W, Legoy MD. Enzyme Microb Technol 2002;31:609.
(b) Jacobsen EE, Hellemond EV, Riise Moen A, Vazquez Prado LC, Anthonsen T. Tetrahedron Lett 2002;44:8453.
[2] (a) Enaud E, Humeau C, Piffaut B, Girardin M. J Mol Catal B 2004;27:1.
(b) Skupinska KA, McEzchern EJ, Baird IR, Skerli RT, Bridger GJ. J Org Chem 2004;68:3546.
[3] (a) Oosterom MWV, Rantwijk FV, Sheldon RA. Biotechnol Bioeng 1996;49:328.
(b) Danieli B, Bertario A, Carrea G, Redigolo B, Secundo F, Riva S. Helv Chim Acta 1996;76:2981.
(c) Danieli B, Luisetti M, Steurer S, Michelitsch A, Likussar W, Riva R, et al. J Nat Prod 1996;62:670.
[4] (a) Teng RW, Ang CS, McManus D, Armstrong D, Mau SL, Bacic A. Tetrahedron Lett 2004;44:5661.
(b) Teng RW, Ang CS, McManus D, Armstrong D, Mau S, Bacic A. Helv Chim Acta 2004;87:1860.
[5] Teng RW, Bui TKA, McManus D, Armstrong D, Mau SL, Bacic A. Biocatal Biotransform 2005;23:109.
[6] Rizk AM, Hammouda FM, El-Missiry MM, Radwan HM, Evans FJ. Phytochemistry 1985;24:1605.
[7] Adolf W, Chanai S, Hecker EJ. J Sci Soc Thai 1983;9:81.
[8] Gillespie SK, Zhang XD, Hersey P. Cancer Res 2004;3:1651.
[9] Hampson P, Chahal H, Khanim F, Hayden R, Mulder A, Assi LK, et al. Blood 2005;106:1362.
[10] (a) Kedei N, Lundberg DJ, Toth A, Welburn P, Garfield SH, Blumberg PM. Cancer Res 2004;64:3243.
(b) Ogbourne SM, Suhrbier A, Jones B, Cozzi SJ, Boyle GM, Morris M, et al. Cancer Res 2004;64:2833.
[11] Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, et al. J Natl Cancer Inst 1990;82:1113.
[12] Marco JA, Sanz-Cervera JF, Yuste A. Phytochemistry 1997;45:563.
[13] Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, et al. Am J Physiol Lung Cell Mol Physiol 2000;279:L429.
[14] Mellor H, Parker P. Biochem J 1998;332:281.