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Chemo-enzymatic preparation of resveratrol derivatives

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Abstract

Regioselective derivatisation of resveratrol (**1**) at positions 3, 5 or 4′ was achieved by a chemo-enzymatic procedure based on standard chemical reactions and esterification or alcoholysis in organic solvents catalysed by the commercially available *Pseudomonas cepacia* (PcL) and *Candida antarctica* (CaL) lipases.

Keywords: Resveratrol; Lipase; Regioselective acylation

1. Introduction

Many natural stilbenoids possess interesting biological activities. Well-known among them, is resveratrol (**1**) (*trans*-3,5,4′-trihydroxystilbene), a metabolite found in *Vitis* spp. and in a variety of other plants and food products. Resveratrol is a potent phytoalexin [1] elicited in response to fungal infections [2] and it has been the subject of a number of pharmacological studies. [3] In particular, **1** is reported as highly antioxidant [4–6], coronary vasodilator [7], inhibitor of platelet aggregation also in vivo [8] and one of the cardioprotective phenolics from red wine [9,10]. It has also shown anti-microbial [11], anti-fungal [12], anti-leukaemic [13] and hepatoprotective [14] activity. It is an inhibitor of protein tyrosine kinase [15] and DNA polymerase [16]; other studies support its anti-inflammatory activity [17] and in particular its possible effect on the COX-2 enzyme [18,19]. Finally, **1** was found to have cancer chemo-protective activity in assays representing three major stages in carcinogenesis, namely anti-initiation, anti-promotion and anti-progression [20]. Resveratrol and other related stilbenoids have also been evaluated for their ability to act as radical scavengers and prevent lipid peroxidation [21]. Also, the resveratrol 3-*O*-β-D-glucopyranoside (piceid) and 4′-*O*-β-D-glucopyranoside (resveratroloside) have been reported as bioactive compounds [15,22].

The important biological properties of resveratrol and related compounds prompted us to carry out the synthesis of partial esters of **1**. These analogues may be useful models for studying the structure–activity relationship, in particular as regards to their amphiphilic and lipophilic properties. In fact, the efficiency of polyphenols as potential therapeutic agents could be related to their lipophilic properties and consequently to their ability to penetrate into the cell [23]. They may also be employed as key intermediates for the synthesis of more complex stilbenoid derivatives.

Regioselective derivatisation of polyhydroxylated compounds based on conventional chemical reactions may cause some difficulties. These have been effectively resolved in many cases by the use of

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biocatalysed reactions. In particular, regioselective acylation or deacylation catalysed by lipases or proteases in organic solvents have been applied to carbohydrates [24] as well as to other natural polyhydroxycompounds [25,26] and, to a lesser extent, to polyphenolic compounds [27–31].

This paper deals with chemo-enzymatic procedures to obtain resveratrol derivatives selectively acylated at positions 3, 5 and 4′, by standard chemical reactions and esterification/alcoholysis catalysed by commercially available lipases, namely *Pseudomonas cepacia* (PcL) and *Candida antarctica* (CaL), as detailed below.

2. Experimental

2.1. General methods

Fast atom bombardment mass spectra (FAB-MS) were recorded on a Kratos MS 50 instrument using 3-nitrobenzylalcohol (NBA) as matrix. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 500 MHz using Varian Unity Inova spectrometer and performed at constant temperature (27 °C) in deuterated methanol (CD_3OD) or chloroform ($CDCl_3$). ^{1}H NMR resonances were assigned on the basis of literature data and chemical shift analysis.

Resveratrol (**1**) was purchased from Sigma. Proteases from *Aspergillus orizae*, lipases from *A. niger*, *C. cylindracea*, *Mucor javanicus* and wheat germ were purchased from Sigma. *P. cepacia* lipase was a gift from Amano. *C. antarctica* (Novozym 435) and *M. miehei* lipases were a gift from Novo Nordisk.

The samples were incubated in a shaker at 40 °C at 400 rpm, along with its respective control (sample with no enzyme). No reaction took place in the absence of the enzyme. All reactions were monitored by TLC at regular time intervals. At the end of the incubation step reactions were quenched by filtering off the enzyme and the filtrates were evaporated in vacuo.

Thin layer chromatography (TLC) was carried out on Merck 60 F_{254} plates using cerium sulphate and phosphomolybdic acid as chromogenic reagents.

Column chromatography was performed by flash chromatography on silica gel DIOL 40–63 μm (Merck) or using the Buchi-Biotage systems FLASH 12^{TM} or FLASH 40^{TM}, equipped with pre-packed silica gel cartridges 12S, 12M or 40S, according to the total amount of crude mixture.

2.2. Preliminary screening: esterification

The enzymatic esterifications were carried out in vials in which the enzyme of chosen (lipases from *A. niger*, *C. cylindracea*, *M. javanicus*, *P. cepacia*, *C. antarctica*, *Mucor miehei* and wheat germ, 5 mg/ml) was added to a solution of **1** (5 mg/ml, 0.02 mmol) in an organic solvent [*t*-amyl alcohol, dioxane, tetrahydrofurane (THF), 2-methoxyethyl ether or 1,2-dichloropropane] containing vinyl acetate (0.02 ml, 0.2 mmol). The reactions were incubated at 40 °C under shaking at 400 rpm. At determined times aliquots of the reaction mixtures were analysed by NMR to determine the substrate conversion.

2.3. Preliminary screening: alcoholysis

To carry out the enzymatic alcoholysis the above cited lipases were added to a solution of **3** in three different solvents, namely, THF, dichloromethane (CH_2Cl_2), *t*-butylmethyl ether (*t*-BME). *n*-Butanol (*n*-BuOH, 0.2 mmol) was added and the suspensions incubated at 40 °C under shaking at 400 rpm. As above, reaction mixtures were analysed by NMR to determine the substrate conversion.

2.4. Enzymatic esterification of resveratrol (*1*)

Novozym 435 (500 mg) was added to a solution of **1** (500 mg, 2.19 mmol) in *t*-amyl alcohol (10 ml) containing vinyl acetate (2 ml, 21.6 mmol) and the suspension was kept at 40 °C under shaking (400 rpm). After 90 h incubation, the reaction was quenched by filtering off the enzyme and the filtrate was taken to dryness under reduced pressure. The residue was purified by flash chromatography on pre-packed silica gel cartridge (from 1 to 3% di-MeOH/CH_2Cl_2) to give 270 mg of **2** and 210 mg of unreacted **1**.

4′-*O*-Acetylresveratrol (**2**): FAB-MS *m/z* 271 $[M+H]^+$; ^{1}H NMR (CD_3OD) δ 7.54 (d, 2H, $J = 8.7$ Hz, H-2′, H-6′), 7.07 (d, 2H, $J = 8.7$ Hz, H-3′, H-5′), 7.03 (d, 1H, $J = 16.3$ Hz, H-α), 6.96 (d, 1H, $J = 16.3$ Hz, H-β), 6.49 (d, 2H, $J = 2.0$ Hz, H-2, H-6), 6.19 (t, 1H,

$J = 2.0$ Hz, H-4), 2.27 (s, 3H, 4′-OAc). Anal. Calcd. for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22; O, 23.68. Found: C, 71.05; H, 5.17; O, 23.78.

2.5. Enzymatic alcoholysis of 3,5,4′-tri-O-acetylresveratrol *3*

The peracetyl derivative, 3,5,4′-tri-*O*-acetylresveratrol (**3**), was prepared in standard conditions using acetic anhydride in pyridine. FAB-MS and ^{1}H NMR spectra of **3** are in agreement with those reported in the literature. [32] A solution of **3** (500 mg, 1.41 mmol) in *t*-BME (25 ml) was added to *n*-BuOH (2 ml, 21.8 mmol) and *C. antarctica* lipase (500 mg) and the mixture was shaken (400 rpm) at 40 °C. After 15 min the reaction was quenched by filtering off the enzyme and the filtrate was evaporated in vacuo. The residue was subjected to flash chromatography on pre-packed silica gel cartridge (from 1 to 3% MeOH/CH_2Cl_2), affording 102 mg of **4**, and 17 mg of **5**. From work-up also 340 mg of unreacted **3** were recovered.

3,5-Di-*O*-acetylresveratrol (**4**): FAB-MS *m/z* 313 $[M + H]^+$; ^{1}H NMR ($CDCl_3$) δ 7.37 (d, 2H, $J =$ 7.9 Hz, H-3′, H-5′), 7.09 (d, 2H, $J = 1.5$ Hz, H-2, H-6), 7.01 (d, 1H, $J = 16.5$ Hz, H-β), 6.86 (d, 1H, $J = 16.5$ Hz, H-α), 6.81 (d, 2H, $J = 7.9$ Hz, H-2′, H-6′), 6.79 (bt, 1H, H-4), 2.32 (s, 6H, 3-OAc, 5-OAc). Anal. Calcd. for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16; O, 25.61. Found: C, 69.18; H, 5.03; O, 25.79.

3-*O*-Acetylresveratrol (**5**): FAB-MS *m/z* 271 $[M + H]^+$; ^{1}H NMR (CD_3OD) δ 7.37 (d, 2H, $J = 8.7$ Hz, H-3′, H-5′), 7.03 (d, 1H, $J = 16.2$ Hz, H-α), 6.86 (d, 1H, $J = 16.2$ Hz, H-β), 6.80 (bd, 1H, H-6), 6.77 (d, 2H, $J = 8.7$ Hz, H-2′, H-6′), 6.72 (bd, 1H, H-2), 6.39 (bt, 1H, H-4), 2.26 (s, 3H, 3-OAc). Assignment for H-2 and H-6 was aided by NOEDS. In particular, irradiation on 3-OAc caused enhancement of H-4 (15%) and H-2 (13%) signals; conversely irradiation on H-2 intensified 3-OAc (17%) signal. Anal. Calcd. for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22; O, 23.68. Found: C, 70.99; H, 5.33; O, 23.68.

The same reaction, carried out for 2 h in the presence of *P. cepacia* lipase, afforded **4** as a single product. This was purified by flash chromatography on pre-packed silica gel cartridge (1% MeOH/CH_2Cl_2) to give 408 mg of **4**.

2.6. Enzymatic alcoholysis of 3,5,4′-tri-O-palmitoylresveratrol *6*

The tripalmitoyl derivative **6** was prepared from **1** using palmitoyl chloride in triethylammine, according to standard chemical procedures.

,5,4′-Tri-*O*-palmitoylresveratrol (6): FAB-MS *m/z* 943 $[M + H]^+$; ^{1}H NMR ($CDCl_3$), stilbenoid moiety: δ 7.49 (d, 2H, $J = 8.5$ Hz, H-2′, H-6′), 7.10 (d, 2H, $J = 2.0$ Hz, H-2, H-6), 7.09 (d, 2H, $J = 8.5$ Hz, H-3′, H-5′), 7.06 (d, 1H, $J = 16.0$ Hz, H-α), 6.98 (d, 1H, $J = 16.0$ Hz, H-β), 6.81 (t, 1H, $J = 2.0$ Hz, H-4); 3,5-palmitoyl chains: 2.56 (t, 4H, $J = 7.5$ Hz, H-2), 1.76 (p, 4H, $J = 7.5$ Hz H-3), 1.42–1.25 (bm, 72 H, overlapped with 4′-chain signals), 0.89 (t, 9H, $J = 7.5$ Hz, H-16 overlapped with 4′-chain signal); 4′-palmitoyl chain: 2.35 (t, 2H, $J = 7.5$ Hz, H-2), 1.64 (p, 2H, $J = 7.0$ Hz, H-3), see above for overlapped signals. Anal. Calcd. for $C_{62}H_{102}O_6$: C, 78.93; H, 10.90; O, 10.17. Found: C, 78.51; H, 10.56; O, 10.93.

n-BuOH (0.7 ml, 7.63 mmol) and CaL (500 mg) were added to a solution of the substrate (500 mg, 0.53 mmol of 6) in *t*-BME (10 ml) and the mixture was shaken (400 rpm) at 40 °C. At the end of the incubation (48 h) the reaction was quenched as usual. The residue was purified by flash chromatography on pre-packed silica gel cartridge (80% CH_2Cl_2/*n*-hexane) to give 120 mg of **7** and 418 mg of **6**.

3,5-Di-*O*-palmitoylresveratrol (**7**): FAB-MS *m/z* 705 $[M + H]^+$; ^{1}H NMR ($CDCl_3$), stilbenoid moiety: δ 7.37 (d, 2H, $J = 8.5$ Hz, H-3′, H-5′), 7.07 (d, 2H, $J = 2.0$ Hz, H-2, H-6), 7.01 (d, 1H, $J = 16.5$ Hz, H-α), 6.87 (d, 1H, $J = 16.5$ Hz, H-β), 6.82 (d, 2H, $J = 8.5$ Hz, H-2′, H-6′), 6.77 (t, 1H, $J = 2.0$ Hz, H-4); 3,5-palmitoyl chains: 2.55 (t, 2H, $J = 7.5$ Hz, H-2), 1.74 (p, 4H, $J = 7.0$ Hz, H-3), 1.42 - 1.25 (bm, 49H), 0.88 (t, 6H, $J = 6.5$ Hz, H-16). Anal. Calcd for $C_{46}H_{72}O_5$: C, 78.36; H, 10.29; O, 11.35. Found: C, 78.41; H, 10.22; O, 11.37.

2.7. Enzymatic alcoholysis of 3,5-di-O-palmitoyl-4′-O-acetylresveratrol *8*

The above cited acetate **2** was treated in standard conditions with palmitoyl chloride in triethylammine to give the triester **8**.

3,5-Di-*O*-palmitoyl-4′-*O*-acetylresveratrol (**8**): FAB-MS *m/z* 747 $[M + H]^+$; ^{1}H NMR ($CDCl_3$),

stilbenoid moiety: δ 7.49 (d, 2H, $J = 9.0$ Hz, H-3′, 5′), 7.10 (d, 2H, $J = 2.0$ Hz, H-2, 6), 7.09 (d, 2H, $J = 9.0$ Hz, H-2′, 6′), 7.05 (d, 1H, $J = 15.9$ Hz, H-α), 6.95 (d, 1H, $J = 15.9$ Hz, H-β), 6.80 (t, 1H, $J = 2.0$ Hz, H-4); 3,5-palmitoyl chains: 2.55 (t, 4H, $J = 7.5$ Hz, H-2), 1.75 (p, 4H, $J = 7.0$ Hz, H-3), 1.42–1.25 (m, 48 H), 0.88 (t, 6H, $J = 6.5$ Hz, H-16); 4′-acetyl: 2.31 (s, 3H, 3-OAc). Anal. Calcd for $C_{48}H_{74}O_6$: C, 77.17; H, 9.98; O, 12.85. Found: C, 77.21; H, 9.91; O, 12.88.

n-BuOH (1 ml, 11 mmol) and PcL (500 mg) were added to a solution of **8** (500 mg, 0.67 mmol) in *t*-BME (10 ml) and the mixture was shaken (400 rpm) at 40 °C. At the end of the incubation (9 h), after quenching, the crude product was purified by flash chromatography on pre-packed silica gel cartridge (80% CH_2Cl_2/*n*-hexane) and gave 235 mg of **7** and 228 mg of **8**.

*2.8. Enzymatic alcoholysis of 3,5-di-O-acetyl-4′-O-palmitoylresveratrol **9***

The diacetate **4** was esterified in standard conditions with palmitoyl chloride in triethylammine to obtain **9**. This substrate, treated as for the above reported alcoholysis in the presence of PcL, gave the diacetate **4** as main product after 2 h.

3,5-Di-*O*-acetyl-4′-*O*-palmitoylresveratrol (**9**): FAB-MS *m/z* 551 $[M + H]^+$; ^{1}H NMR ($CDCl_3$), stilbenoid moiety: δ 7.48 (d, 2H, $J = 8.7$ Hz, H-2′, H-6′), 7.11 (d, 2H, $J = 1.9$ Hz, H-2, H-6), 7.08 (d, 2H, $J = 8.7$ Hz, H-3′, H-5′), 7.06 (d, 1H, $J = 16.0$ Hz, H-α), 6.97 (d, 1H, $J = 16.0$ Hz, H-β), 6.82 (t, 1H, $J = 1.9$ Hz, H-4); 4′-palmitoyl chain: 2.55 (t, 2H, $J = 7.5$ Hz, H-2), 1.76 (p, 4H, $J = 7.0$ Hz, H-3), 1.42–1.25 (m, 22 H), 0.89 (t, 3H, $J = 7.5$ Hz, H-16); 3(5)-acetyl: 2.31 (s, 6H, 3-OAc, 5-OAc). Anal. Calcd for $C_{34}H_{46}O_4$: C, 74.15; H, 8.42; O, 17.43. Found: C,74.03; H, 8.39; O, 17.58.

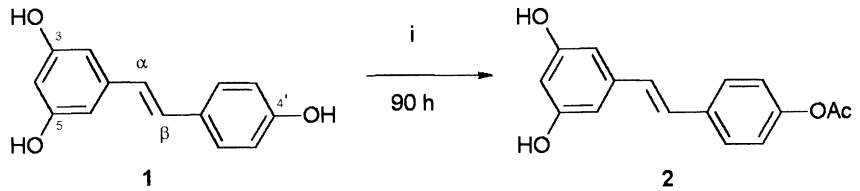
3. Results and discussion

Eight commercial enzymes of microbial and plant origin, namely lipases from *A. niger*, *C. antarctica*, *C. cylindracea*, *M. javanicus*, *M. miehei*, *P. cepacia* and wheat germ, as well as a protease from *A. orizae*, were used for a preliminary screening of biocatalysed esterification of **1** and alcoholysis of **3** in suitable organic solvents. Esterifications (acylation of **1**) were carried out with vinyl acetate in *t*-amyl alcohol, dioxane, THF, 2-methoxyethyl ether and 1,2-dichloropropane. These latter two solvents were discarded because of the poor solubility of the substrate and absence of conversion, while *t*-amyl alcohol was selected for its good solubility and observed conversion in the presence of *C. antarctica* lipase (CaL). Alcoholysis of 3,5,4′-tri-*O*-acetylresveratrol **3** were run with *n*-BuOH in THF, CH_2Cl_2, and *t*-BME. This latter appeared the more convenient solvent, showing deprotection both with *C. antarctica* and *P. cepacia* lipases (PcL). On the basis of these preliminary reactions, preparative procedures were carried out for the biocatalysed synthesis of regioselectively acylated derivatives of **1**, as detailed in Section 3.1.

*3.1. Enzymatic esterification of resveratrol **1***

The reaction of **1** with vinyl acetate in the presence of CaL (Scheme 1), was rather slow, but afforded essentially a single acylation product, 4′-acetylresveratrol (**2**) obtained with 40% yield after 90 h, isolated by flash-chromatography and characterised by FAB-MS and ^{1}H NMR spectroscopy. Further prolongation of the reaction time (6 days) did not allow improvement of the reaction yield.

Thus resveratrol, in the reported conditions, is a weak substrate for CaL, though this enzyme shows high selectivity for the hydroxyl group at 4′ position.



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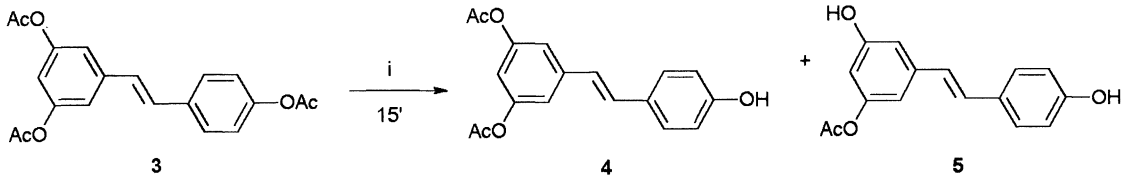
Scheme 1. (i) *C. antarctica* lipase, vinyl acetate, and *t*-amyl alcohol.



Scheme 2. (i) *C. antarctica* lipase, *n*-butanol, and *t*-butylmethyl ether.

*3.2. Enzymatic alcoholysis of 3,5,4′-triacetylresveratrol **3***

The peracetate **3**, prepared from **1** according to a standard acetylation procedure, was subjected to CaL catalysed alcoholysis with *n*-BuOH. This reaction (Scheme 2) was fast and afforded after a few minutes a first deprotection product, subsequently isolated and characterised as 3,5-diacetylresveratrol **4**. In a 15 min run, the reaction mixture showed two deacylation products and the conversion was evaluated as 30%. These products were isolated by flash-chromatography and characterised by FAB-MS and ^{1}H NMR as **4** and the 3-acetylresveratrol **5**, respectively, obtained in 6:1 ratio. Prolonging the reaction time to 45 min did not allow improvement of the yield of **4**, due to its deacylation to **5** and subsequently **1**.

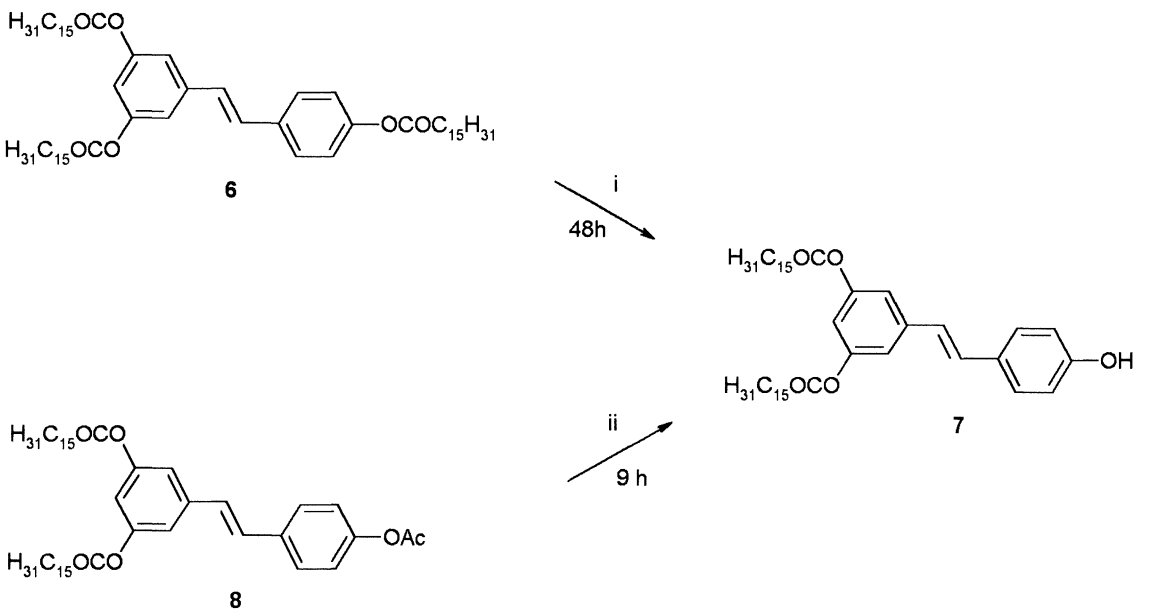
The same reaction, carried out in the presence of PcL, resulted slower but highly selective: after 2 h, deprotection at 4′ was almost complete and allowed **4** in 95% yield recovered. Even with prolonged reaction time (24 h), the presence of more polar products due to further deprotection was negligible. This showed that PcL recognises preferentially the monohydroxylated ring in the stilbenoid structure of **1**.

*3.3. Chemo-enzymatic preparation of 3,5-dipalmitoylresveratrol **7***

In order to test the alcoholysis also for long-chain acyl-derivatives of resveratrol, we prepared the 3,5,4′-tripalmitoylresveratrol (**6**) by standard acylation of **1** with palmitoyl chloride. The triester **6** was treated with *n*-butanol in the presence of PcL, but no reaction product was obtained even prolonging time to 5 days. Thus, we resorted to the reaction in the presence of CaL. After ca. 40 h, a product, more polar than the substrate **6**, was obtained with a conversion rate of 34%. On prolonging the reaction time, further polar products were generated, among them **1**, and this suggested to stop the reaction after 48 h (Scheme 3i). The crude reaction mixture was subjected to flash-chromatography and afforded a main product (yield 30%) characterised spectroscopically as 3,5-dipalmitoylresveratrol (**7**). We tried also a different chemo-enzymatic way to obtain **7**. The 4′-acetylresveratrol **2** was esterified in 3,5 positions with palmitoyl chloride. This compound (3,5-dipalmitoyl-4′-acetylresveratrol **8**) was subjected to PcL catalysed alcoholysis with *n*-BuOH, and proved to be more reactive than **6**, giving **7** in a significantly shorter time (9 h, conversion 54%), as a single product (Scheme 3ii). This latter could be conveniently purified through flash-chromatography with a satisfactory yield (47%). Thus, this second way allowed shortening of the reaction time and a slight enhancement of the final yield.

It is worth noting, here, that a similar strategy to obtain the 4′-palmitoylresveratrol (based on the observed faster CaL-catalysed removal of acyl groups in **3**, with respect to **6**) revealed unsuccessful: indeed, when 3,5-diacetyl-4′-palmitoylresveratrol **9**, prepared from the 3,5-diacetate **4** by chemical esterification, was subjected to alcoholysis in *t*-BME catalysed by CaL, gave after just 2 h the diacetate **4** as the main product, clearly indicating that regioselectivity is the dominating factor in this last reaction.

In conclusion, the reported reactions show useful applications of lipases in organic solvents in the preparation of regioselectively acylated derivatives of the important stilbenoid resveratrol. In particular, a high selectivity for the 4′ position was observed both for enzymatic esterification and alcoholysis. Development of these enzymatic or chemo-enzymatic procedures could allow the preparation of derivatives from bioactive polyhydroxystilbenes bearing different acyl groups in various positions, suitable for structure–activity relationship studies and



Scheme 3. (i) *C. antarctica* lipase, *n*-butanol, and *t*-butylmethyl ether; (ii) *P. cepacia* lipase, *n*-butanol, and *t*-butylmethyl ether.

possibly improvement of their known pharmacological properties.

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