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Use of *Mucor miehei* lipase in the preparation of long chain 3-*O*-acylcatechins

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Abstract

Long chain 3-*O*-acylcatechins were prepared in high yield by alcoholysis with *n*-butanol of the corresponding pentaacylderivatives in the presence of lipase from *Mucor miehei* (immobilised, Lipozyme® IM). In an alternative procedure, the mixed ester, tetraacetyl-3-*O*-acylcatechin, was synthesised and used as substrate for the same alcoholysis process that proceeds with higher reaction rate. The obtained 3-*O*-acyl derivatives are more lipophilic than the parent catechin and thus suitable for a possible application of their antioxidative properties in hydrophobic matrices.

Keywords: Flavonoids; Enzyme reaction; Regioselection; Esters

1. Introduction

Flavonoids belong to a family of natural polyhydroxylated diphenylpyrane derivatives, ubiquitous in the plant kingdom, which includes flavanols, flavanones, anthocyanidins, flavanes and flavonols. The spectacular increase of interest in their biological effects is mainly due to their cardioprotective [1] and anticarcinogenic action [2,3], attributed to their antioxidant potential against free radicals [4]. Among the flavanols, particularly attractive are the catechins, including (+)-catechin, (+)-gallocatechin, (−)-epicatechin, (−)-epigallocatechin and their gallate esters, which are constituents of teas (black and green) and red wine, and possibly responsible for the intriguing "French paradox".

In practical exploitation of their antioxidant properties, for instance as protective agents for fats and oils against aerial oxidation and perhaps also in cosmetology, a limit is set by the hydrophilic nature of these compounds that may hinder solubility in lipidic matrices or penetration in the skin. This solubility problem can be overcome by introducing a hydrophobic group in the molecule, without modifying its antioxidant capacity, mainly due to the *ortho*-hydroxyls in the B ring [5,6]. Good candidates for these uses are the long-chain 3-*O*-acyl esters of (+)-catechin, whose synthesis through esterification catalysed by esterase from *Aspergillus*

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niger or *Streptomyces rochei* has been reported (Mitsui Norin, Tokyo) [7]. However, the unsatisfactory yields (less than 20%) and the use of expensive esterases prompted us to search for a better method and in the present paper we wish to report the results obtained.

2. Experimental

2.1. Material and methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution, unless otherwise stated, at 250.13 and 62.9 MHz, respectively on a Bruker AMX-250 instrument using TMS as internal reference. Optical rotations were measured on a DIP 135 JASCO instrument. Lipases from *Candida cylindracea* and *Pseudomonas cepacia* were obtained from Amano International Enzyme. Porcine pancreas lipase was from Sigma. Lipozyme® IM (immobilised lipase from *Mucor miehei*) and Novozyme® 435 (immobilised lipase from *C. antarctica*) are registered marks from Novo Nordisk. Column chromatography was performed on silica-gel or Lichropep Si-Diol 40–63 μm (Merck); analytical TLC was carried out on Merck silica gel 60-F_{254} precoated glass plates and compounds were visualized by spraying with molybdophosphoric acid.

2.2. General procedure for synthesis of penta-O-acylcatechins 2–4

To a solution of (+)-catechin (0.50 g, 1.72 mmol) in *t*-butylmethyl ether (*t*-BME), acid anhydride (17.2 mmol) or acid chloride (17.2 mmol) and triethylamine (17.2 mmol) were added and the mixture stirred at room temperature until TLC analysis showed the complete conversion of substrate and formation of a single product. The reaction mixture was then extracted with 1 N HCl and the organic layer washed with aq Na_2CO_3 and H_2O. The pooled extracts were dried over Na_2SO_4 and then taken to dryness to give pentaacylcatechins **2**–**4**.

3,5,7,3′,4′-Penta-*O*-propionylcatechin (**2**) was obtained in 94% yield as a colourless oil; $[\alpha]_D$ −2.2 (*c* 2.8, C_6H_6); ^{1}H NMR δ 1.05 (3H, t, $J = 7.5$ Hz, CH_3–), 1.26 (12H, t, $J = 7.5$, $4 \times CH_3$–), 2.28 (2H, q, $J = 7.5$ Hz, CH_3CH_2–), 2.58 (8H, q, $J = 7.5$ Hz, $4 \times CH_3CH_2$–), 2.67 (dd, 1H, $J = 6.5$ and 16.5 Hz, H-4a), 2.90 (dd, 1H, $J = 5.0$ and 16.5 Hz, H-4b), 5.15 (1H, d, $J = 6.2$ Hz, H-2), 5.28 (1H, ddd, $J = 5.0$, 6.2 and 6.5 Hz, H-3), 6.61 (1H, d, $J = 2.2$ Hz, H-6), 6.67 (1H, d, $J = 2.2$ Hz, H-8), 7.19–7.29 (3H, m, H-2′, 5′ and 6′); ^{13}C NMR δ 8.8, 9.1, 24.0, 27.4, 27.7, 68.2, 77.8, 107.5, 108.7, 110.1, 121.8, 123.6, 124.4, 136.0, 142.2, 149.4, 149.9, 154.4, 171.5, 171.9, 172.5, 173.6. Anal. Calcd. for $C_{30}H_{34}O_{11}$: C, 63.15; H, 6.00. Found: C, 62.96; H, 5.97.

3,5,7,3′,4′-Penta-*O*-valerylcatechin (**3**) was prepared in 92% yield as a glassy oil; $[\alpha]_D$ −3.0 (*c* 1.2, $CHCl_3$); ^{1}H NMR δ 0.88 (3H, t, $J = 7.5$ Hz, CH_3–), 0.98 (12H, t, $J = 7.5$, $4 \times CH_3$–), 1.25 (2H, m, $-CH_2$–), 1.46 (10H, m, $-CH_2$–), 1.65 (8H, m, $-CH_2$–), 2.27 (2H, t, $J = 7.5$ Hz, $-OCOCH_2$), 2.56 (8H, t, $J = 7.5$ Hz, $4 \times OCOCH_2$–), 2.66 (dd, 1H, $J = 6.5$ and 16.5 Hz, H-4a), 2.89 (dd, 1H, $J = 5.0$ and 16.5 Hz, H-4b), 5.15 (1H, d, $J = 6.5$ Hz, H-2), 5.28 (1H, ddd, $J = 5.0$, 6.5 and 6.5 Hz, H-3), 6.59 (1H, d, $J = 2.2$ Hz, H-6), 6.66 (1H, d, $J = 2.2$ Hz, H-8), 7.19–7.29 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR δ 13.6, 14.1, 21.4, 22.0, 24.0, 26.7, 26.9, 33.8, 34.0, 68.1, 77.7, 107.5, 108.7, 110.1, 121.8, 123.6, 124.3, 136.0, 142.2, 149.4, 149.9, 154.4, 170.9, 171.2, 171.8, 173.0. Anal. Calcd. for $C_{40}H_{54}O_{11}$: C, 67.58; H, 7.66. Found: C, 67.92; H, 7.69.

3,5,7,3′,4′-Penta-*O*-palmitoylcatechin (**4**) was obtained in 90% yield as a white powder, mp 59–61°C; $[\alpha]_D$ −1.8 (*c* 1.8, $CHCl_3$); ^{1}H NMR δ 0.91 (15H, t, $J = 7.0$ Hz, $5 \times CH_3$), 1.29 (120H, m, $5 \times (CH_2)_{12}$), 1.75 (10H, m, $5 \times OCOCH_2CH_2$), 2.25 (2H, t, $J = 7.5$ Hz, $OCOCH_2$), 2.55 (8H, t, $J = 7.5$ Hz, $4 \times OCOCH_2$), 2.65 (1H, dd, $J = 6.5$ and 16.5 Hz, H-4a), 2.89 (1H, dd, $J = 5.0$ and 16.5 Hz, H-4b), 5.15 (1H, d, $J = 6.2$ Hz, H-2), 5.28 (1H, ddd, $J = 5.0$, 6.2

and 6.5 Hz, H-3), 6.59 (1H, $J = 2.2$ Hz, H-6), 6.66 (1H, d, $J = 2.2$ Hz, H-8), 7.17–7.29 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR δ 14.1, 22.7, 24.9, 29.2, 29.4, 31.9, 34.1, 68.1, 77.7, 107.5, 108.7, 110.1, 121.8, 123.7, 124.3, 136.0, 142.2, 149.4, 149.9, 154.4, 170.8, 171.2, 171.8, 173.0. Anal. Calcd. for $C_{95}H_{164}O_{11}$: C, 76.97; H, 11.15. Found: C, 76.74; H, 11.08.

*2.3. Preliminary experiments of lipase catalysed alcoholysis of **3***

Lipase of choice (10 mg/ml) was added to a solution of **3** (10 mg/ml) in an organic solvent containing *n*-butanol (10 eqv). The suspension was shaken (300 rpm) at 45°C and the course of the reaction followed by TLC at regular time intervals. The following solvents (THF, *t*-BME and CH_2Cl_2) and lipases (from *C. antarctica*, *C. cylindrica*, *P. cepacia*, *Rizophus javanicus*, *M. miehei* and porcine pancreas lipase) were used. The best results in terms of reaction rate were obtained with *M. miehei* in *t*-BME.

*2.4. Alcoholysis of 3,5,7,3′,4′-penta-O-acylcatechins **2**–**4** (Method A)*

Lipase from *M. miehei* (immobilised, Lipozyme® IM, 0.50 g) was added to a solution of the pentaacylcatechin of choice (0.50 g) in *t*-BME containing *n*-butanol (10 eqv). The suspension was shaken (300 rpm) at 45°C until TLC analysis of the reaction mixture showed the presence of a single product (36–96 h). The reaction was then quenched filtering off the catalyst and the filtrate evaporated to dryness in vacuo. The residue was purified by column chromatography on Si gel (petroleum ether/acetone 7:3) to give the relevant 3-*O*-acylcatechin.

3-*O*-propionylcatechin (**2a**) was isolated in 94% yield as white solid, mp 208°C; $[\alpha]_D = +1.0$ (*c* 0.5, EtOH); ^{1}H NMR (CD_3OD) δ 1.01 (3H, t, $J = 7.5$ Hz, CH_3), 2.25 (2H, q, $J = 7.5$ Hz, CH_2), 2.63 (1H, dd, $J = 7.0$ and 16.5 Hz, H-4a), 2.82 (1H, dd, $J = 5.2$ and 16.5 Hz, H-4b), 4.87 (1H, d, $J = 6.2$ Hz, H-2), 5.23 (1H,ddd, $J = 5.2$, 6.2 and 7.0 Hz, H-3), 5.92 (1H, d, $J = 2.2$ Hz, H-6), 5.97 (1H, d, $J = 2.2$ Hz, H-8), 6.69–6.83 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR (CD_3OD) δ 9.3, 24.9, 28.4, 70.9, 79.5, 95.5, 96.4, 99.6, 114.6, 116.0, 119.4, 131.1, 146.3, 156.5, 157.5, 158.1, 175.2. Anal. Calcd. for $C_{18}H_{18}O_7$: C, 62.42; H, 5.24. Found: C, 62.54; H, 5.29.

3-*O*-valerylcatechin (**3a**) was obtained in 90% yield as clear syrup; $[\alpha]_D = +2.5$ (*c* 0.7, EtOH); ^{1}H NMR (CD_3OD) δ 0.85 (3H, t, $J = 7.2$ Hz, CH_3), 1.17 (2H, m, CH_2), 1.44 (2H, m, CH_2), 2.23 (2H, t, $J = 7.0$ Hz, OC-OCH_2), 2.65 (1H, dd, $J = 7.0$ and 16.3 Hz, H-4a), 2.81 (1H, dd, $J = 5.2$ and 16.3 Hz, H-4b), 4.91 (1H, d, $J = 6.4$ Hz, H-2), 5.21 (1H, ddd, $J = 5.2$, 6.4 and 7.0 Hz, H-3), 5.90 (1H, d, $J = 2.2$ Hz, H-6), 5.96 (1H, d, $J = 2.2$ Hz, H-8), 6.68–6.82 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR (CD_3OD) δ 13.9, 22.9, 25.2, 28.2, 34.9, 70.9, 79.6, 95.4, 96.4, 99.6, 114.7, 116.1, 119.5, 130.0, 146.4, 156.1, 157.0, 158.1, 175.0. Anal. Calcd. for $C_{20}H_{22}O_7$: C, 64.16; H, 5.92. Found: C, 64.48; H, 5.97.

3-*O*-Palmitoylcatechin (**4a**) was isolated in 70% yield as white solid, mp 136–8°C; $[\alpha]_D = +7.2$ (*c* 0.5, EtOH); ^{1}H NMR (CD_3OD) δ 0.95 (3H, bt, CH_3), 1.33 (24H, m, $(CH_2)_{12}$), 1.62 (2H, m, $OCOCH_2CH_2$), 2.19 (2H, t, $J = 7.2$ Hz, $OCOCH_2$), 2.62 (1H, dd, $J = 7.0$ and 16.2 Hz, H-4a), 2.84 (1H, dd, $J = 5.2$ and 16.2 Hz, H-4b), 4.88 (1H, d, $J = 6.5$ Hz, H-2), 5.22 (1H, ddd, $J = 5.2$, 6.5 and 7.0 Hz, H-3), 5.90 (1H, d, $J = 2.2$ Hz, H-6), 5.96 (1H, d, $J = 2.2$ Hz, H-8), 6.68–6.82 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR (CD_3OD) δ 14.4, 23.7, 26.0, 30.2, 33.0, 34.9, 70.9, 79.6, 95.4, 96.4, 99.6, 114.7, 116.0, 119.5, 131.0, 146.4, 156.1, 157.5, 158.0, 174.5. Anal. Calcd. for $C_{31}H_{44}O_7$: C, 70.43; H, 8.39. Found: C, 69.88; H, 8.31.

2.5. Preparation of 3-O-palmitoylcatechin (Method B)

To a stirred solution of (+)-catechin (0.50 g, 1.73 mmol) in *t*-BME (100 ml) containing tri-

ethylamine (1.5 ml) Ac_2O (0.6 ml, 6.89 mmol) was added dropwise. The reaction mixture was kept at room temperature for 24 h and then added with aq 1 N HCl. The organic layer was washed with aq $NaHCO_3$, dried over Na_2SO_4 and the solvent evaporated in vacuo. The residue (containing compound **5** in about 65%) was dissolved in *t*-BME and treated with palmitoyl chloride (1.0 ml) and triethylamine (1.0 ml). After 12 h, 1 N HCl was added, the organic layer separated and the aqueous phase extracted twice with *t*-BME. The organic phases were pooled, washed with aq $NaHCO_3$ and dried over Na_2SO_4 to give a residue containing compound **6** as main product. This residue was dissolved in *t*-BME (10 mg/ml) containing Lipozyme® IM (10 mg/ml) and *n*-BuOH (1.6 ml, 17.30 mmol). The suspension was stirred (300 rpm) for 36 h at 45°C, then the catalyst was filtered off and the filtrate evaporated in vacuo. The residue was purified on Si gel column (petroleum ether/acetone 7:3) to afford monoester **4a** (0.82 g, yield 90%).

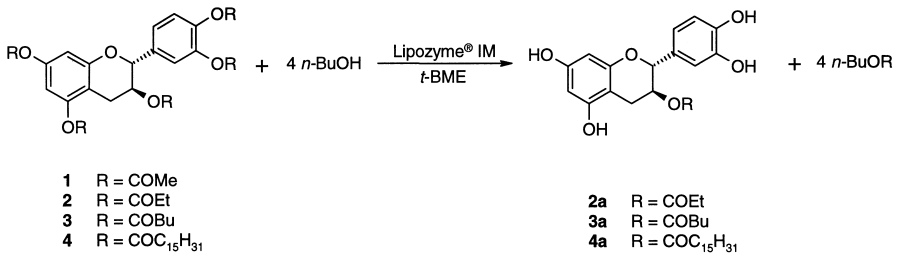
5,7,3′,4′-Tetra-*O*-acetylcatechin (**5**): Acetylation of (+)-catechin (0.50 g) as above gave a residue that was purified (SiO_2, petroleum ether/acetone 7:3) to yield ester **5** (0.51 g, yield 65%), $[\alpha]_D = +0.6$ (c 0.5, EtOH); ^{1}H NMR δ 2.29 (s, 3H, $-CH_3$), 2.33 (9H, $3 \times -CH_3$), 2.65 (1H, $J = 9.5$ and 16.5 Hz, H-4a), 3.00 (1H, $J = 5.5$ and 16.5 Hz, H-4b), 3.96 (1H, ddd, $J = 5.5$, 8.5 and 9.5 Hz, H-3), 4.74 (1H, d, $J = 8.5$ Hz, H-2), 6.57 (1H, d, $J = 2.2$ Hz, H-6), 6.62 (1H, d, $J = 2.2$ Hz, H-8), 7.24–7.38 (3H, m, H-2′, H-5′ and H-6′). Anal. Calcd. for $C_{23}H_{22}O_{10}$: C, 60.26; H, 4.84. Found: C, 60.57; H, 4.88.

5,7,3′,4′-Tetra-*O*-acetyl-3-*O*-palmitoylcatechin (**6**): A solution of **5** (0.50 g, 1.09 mmol) in *t*-BME was added to palmitoyl chloride (0.50 ml) and triethylamine (0.25 ml). After conventional work-up the residue was chromatographed on Si gel (petroleum ether/AcOEt 8:2) to give 0.68 g (yield 90%) of mixed ester **6**, $[\alpha]_D = +72.8$ (*c* 1.35, $CHCl_3$); ^{1}H NMR δ 0.91 (3H, bt, CH_3 palm), 1.28 (24H, bs, $(CH_2)_{12}$), 1.49 (2H, m, $-OCOCH_2CH_2$), 2.25 (2H, m, $-OCOCH_2$), 2.30 (s, 12H, $4 \times -CH_3$), 2.71 (1H, dd, $J = 6.5$ and 16.7 Hz, H-4a), 2.90 (1H, dd, $J = 5.0$ and 16.7 Hz, H-4b), 5.16 (1H, d, $J = 6.2$, H-2), 5.27 (1H, ddd, $J = 5.0$, 6.2 and 6.5 Hz, H-3), 6.62 (1H, d, $J = 2.0$ Hz, H-6), 6.67 (1H, d, $J = 2.0$ Hz, H-8), 7.20–7.29 (3H, m, H-2′, H-5′ and H-6′); ^{13}C NMR ($CDCl_3$): δ 15.4, 21.9, 22.2, 22.3, 24.0, 25.2, 26.2, 30.9, 33.2, 35.4, 69.6, 79.0, 108.8, 110.1, 111.5, 123.1, 125.0, 125.7, 137.5, 143.4, 150.8, 151.2, 155.7, 169.3, 170.2, 171.4, 172.5. Anal. Calcd. for $C_{39}H_{52}O_{11}$: C, 67.22; H, 7.52. Found: C, 67.48; H, 7.55.

3. Results and discussion

It was known from previous work [8] that the C-3 hydroxyl is not recognised by *P. cepacia* lipase either in the esterification of (+)-catechin or in the complementary reaction of alcoholysis with *n*-butanol of penta-*O*-acetylcatechin, **1**. When the latter reaction was carried out in THF/*n*-BuOH mixture, 3-*O*-acetyl-catechin was obtained in high yield.

We decided to apply this lipase-catalyzed alcoholysis procedure to prepare long chain 3-*O*-acylcatechins using the corresponding penta-*O*-acyl derivative as substrate. Catechin pentavalerate **3** was chosen as model substrate for preliminary experiments in view of the intermediate size of its ester chain. However, alcoholysis of **3** with *n*-butanol in the presence *P. cepacia* lipase and using different solvents was unaffective, presumably due to the steric hindrance produced by valeryl groups. Therefore, we resort to the investigation of the alcoholysis of **3** using a set of several lipases from different source (from *C. cylindracea*, *C. antarctica*, *R. javanicus*, *M. miehei* and porcine pancreas), all commercially available. From these lipases, only the one from *M. miehei* (immobilised, Lipozyme® IM) in *t*-BME proved to be very active and was selected for further study.

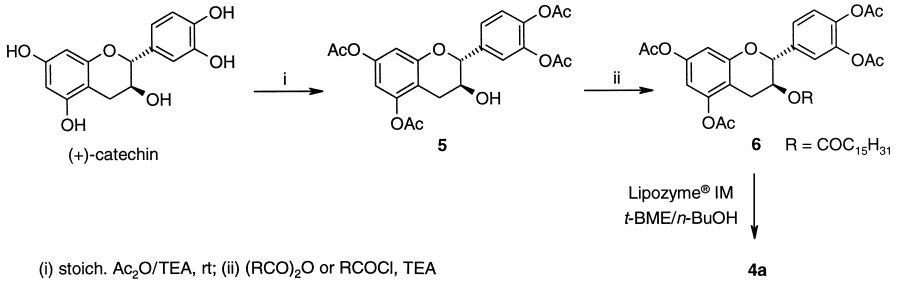


Scheme 1.

The alcoholysis reaction in the presence of Lipozyme® IM was therefore applied to penta-*O*-propionylcatechin, **2**, penta-*O*-valerylcatechin, **3** and penta-*O*-palmitoylcatechin, **4** in a semipreparative scale and the results obtained showed that the reaction rate decreases with increasing chain length, requiring for completion 36 h for the penta-*O*-propionylderivative and about four days for the last two esters. Due to the high regioselectivity of the reaction **2a**, **3a** and **4a**, were obtained in very satisfactory yields (Method A, Scheme 1).

To circumvent the long reaction time and simplify the purification procedure to recover the desidered 3-*O*-acylcatechin from the long-chain *n*-butylester produced in the alcoholysis reaction, we resorted to a different approach, described here for the preparation of ester **4a** (Method B, Scheme 2). In the first step, exploiting the preferential chemical esterification of phenolic hydroxyls, acetylation of (+)-catechin with stoichiometric Ac_2O in the presence of triethylamine allowed to prepare a mixture of partial esters of catechin all possessing a free hydroxyl at C-3, as assessed from ^{1}H NMR analysis of the reaction mixture. After purification on silica gel column the structure of **5** could be assigned to the ester present as principal product (65% yield). In the second step, the whole acetylation mixture was treated with palmitoyl chloride to give the mixed ester **6** as main product and then subjected to alcoholysis with *n*-butanol in the presence of *M. miehei* lipase. Complete deprotection of the hydroxyl groups on the aromatic rings occurred with satisfactory rate, due to the higher reactivity of the acetyl groups, and the reaction was complete after 36 h to give the desired ester **4a** (90% yield).

In conclusion, we have developed a simple and convenient biocatalytic procedure to prepare in high yield catechin esters bearing a long-chain acyl group at C-3 position. Studies aimed at evaluating the possibility to employ



Scheme 2.

these lipophilic compounds as "natural" radical scavengers in food are under investigation and will be published elsewhere.

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